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In vitro comparison of complementary interactions between synthetic linear/branched oligo/poly-L-lysines and tissue plasminogen activator by means of high-performance monolithic-disk affinity chromatography ☆

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Abstract

The recently discovered serine protease called *tissue plasminogen activator* (t-PA) enables efficient dissolution of blood clots. t-PA works by converting plasminogen into its active form, plasmin, dissolving the major component of blood clots, fibrin. The activation of plasminogen by t-PA is enhanced by the presence of fibrin, and this is probably due to the fact that both plasminogen and t-PA possess high affinity binding sites for fibrin. Besides fibrin, fibrin monomers and some fibrin(ogen) degradation products, certain synthetic polymers (for instance, poly-L-lysines) can provide the same stimulation of plasminogen activation. The recently developed high-performance monolithic-disk chromatography, HPMDC, could become the most convenient way to study biological pairs of interest. The inherent speed of HPMDC isolation facilitates the recovery of a biologically active product, since the exposure to putative denaturing influences, such as solvents or temperature, is reduced. The better mass transfer mechanism (convection rather than diffusion) allows to consider only the biospecific reaction as time limiting. The step-by-step modeling of hypothetical affinity pairs between t-PA and different types of oligo/polymer forms of linear and branched lysine derivatives obtained both by initiated polycondensation and solid-phase peptide synthesis using HPMDC seemed to be possible and a quite useful tool. The results of quantitative evaluation of such affinity interactions were compared with those established for natural affinity counterparts to t-PA (monoclonal antibodies, plasminogen, fibrinogen). The role of steric structure of lysine ligands was observed and analyzed. The results allowing to make the practical choice of affinity systems will be used for development of fast and efficient analytical and preparative methods for the downstream processes of recombinant production of this valuable enzyme.

Keywords: Monolithic disks; Affinity chromatography; Fibrinolysis; Peptides; Plasminogen activator; Enzymes; Oligolysines; Polylysines

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1. Introduction

It is well known that the serine protease called tissue plasminogen activator (t-PA) efficiently dissolves blood clots [1–4]. Thus, this protein seems to be extremely useful in clinical practice in the cases of heart attack victims. However, a high therapeutic level of t-PA in blood (in the μg/ml ranger is required to obtain rapid coronary artery reperfusion.

The activation of plasminogen by t-PA is enhanced substantially more by the presence of fibrin than fibrinogen, probably due to the fact that both plasminogen and t-PA possess higher affinity binding sites for fibrin [5–8]. Besides fibrin, fibrin monomers and some fibrin(ogen) degradation products mentioned above, certain synthetic polymers (for instance, poly-L-lysine) can provide the same stimulating plasminogen activation effect [8,9]. It is also known that short GPRP tetrapeptide, an inhibitor of fibrin polymerization, plays an inhibiting role in t-PA-fibrin interaction [10].

The data listed above show that the process of fibrinolysis represents a very complicated network of simultaneous biological events. It is clear that t-PA has a branched set of functional complements with their own, and probably different, affinity to this enzyme. It seems to be possible and quite interesting to investigate all these pairs by separately creating them in vitro. This research would make it a possible to define accurately the properties of complementary pairs with the participation of t-PA. The solved fundamental problem can appear quite useful from a practical side. It is clear that recombinant technology of t-PA production will become a main source of therapeutic amounts of this valuable remedy. It means that such general steps as protein purification will need productive, rapid and robust methods. Affinity chromatography is dominant in this range. In its turn, a crucial point of this method is a choice of the ''right ligand'' complementary to the product to be isolated.

The recently developed high-performance monolithic-disk chromatography, HPMDC [11–14], is quite promising in both scientific and practical regards because of its high capacity and selectivity, combined with low back pressure and short operation times. The affinity mode of HPMDC is likely to overcome many critical disadvantages of conventional affinity chromatography. Most importantly, the better mass transfer mechanism (convection rather than diffusion) allows only the biospecific reaction to be time limiting. The last fact can be used effectively not only in affinity separation processes but also for in vitro modeling of biological events following the forming of complementary functional pairs [15–19].

The results of quantitative analysis of a range of modeled pairs between t-PA and its probable natural and synthetic complements using modem affinity HPMDC are presented in this paper. Taking into account the elevated stability and controlled adsorption capacity, the main attention has been paid to the synthetic t-PA counterparts including linear polymers and peptides of different length and structure. By comparing binding parameters of t-PA with its various possible complements, the data obtained in this research can help illuminate the on t-PA-dependent part of fibrinolysis. Finally, the right affinity ligand for fast and efficient analytical (on-line analysis) and preparative methods for down stream processing of t-PA production can be chosen.

2. Experimental

2.1. Materials and chemicals

Macroporous monolithic disks of 12×3 mm with mean pore size of 1.5 μm and a porosity of 0.63 ml/ml sorbent (standard CIM epoxy disk; CIM stands for convective interactive medium) as well as specially designed cartridges were from BIA Separations (Ljubljana, Slovenia).

Plasminogen and fibrinogen were purchased from Sigma (Germany) and bulk chemicals for buffer preparation were from Fluka (Switzerland). t-PA standards as well as monoclonal anti-t-PA antibodies were kindly donated by Boehringer Ingelheim Pharma (Biberach, Germany).

Diisopropylcarbodiimide (DIC), trifluoromethanesulfonic acid (TFMSA), trifluoroacetic acid (TFA), thioanisole, ethanedithiol, hydroxybenzotriazole (HOBt), triphosgen (TPG) and *p*-toluenesulfonic acid (TosOH) were from Fluka. Dimethylformamide (DMF), dichloromethane (DCM), and triethylamine (TEA) were purchased from Vecton (Russia).

Standard protein and peptide kits for polyacrylamide gel electrophoresis (PAGE) and size-exclusion chromatography (SEC) were purchased from BioRad (USA) and Sigma, respectively. All chemicals used in these methods including those for gels and buffers preparation were from Fluka and Sigma.

Buffers used for immobilization procedures as well as 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 microfilter (Millipore, 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} with 150×4.6- and 250×22-mm 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.

The molecular masses of prepared polymeric derivatives of lysine were verified by sodium dodecylsulfate (SDS)–PAGE carried out using Mini Protean II System (Bio-Rad) with silver staining detection.

Additionally, to evaluate the molecular masses of poly-L-lysines, SEC using a 30×1.5-cm two-adaptor glass column (Pharmacia, Sweden) filled with Sephadex G-200 (Pharmacia) was explored. The column was connected with peristaltic pump (2115 Multiperpex pump, LKB, Bromma, Sweden) and UV detector (2138 Uvicord 5, LKB). The SEC method was also applied to establish the molecular masses of synthesized peptides. In this case, a Beckman System Gold (USA) chromatograph and 150×4-mm TSK 2000 PW column (Toya Soda, Japan) were used.

The ion-exchange mode of HPMDC (IE-HPMDC) was performed for quality control of synthesized linear peptidyl lysines. For this purpose, a cation-exchange CIM SO_3 disk was used as a stationary phase a gradient chromatographic system Gilson (France) combining two piston pumps 303 and 305 and 118 UV–Vis detector.

Affinity HPMDC was carried out using a chromatographic system consisting of a peristaltic pump (2115 Multiperpex pump, LKB), a UV detector (2138 Uvicord S, LKB) and a recorder (2210 Recorder, LKB).

To isolate t-Pa from a Chinese hamster ovary (CHO) cell supernatant, a chromatographic system consisting of Waters (USA), 2690 Alliance separation module, Waters 490 scanning fluorescence 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).

Gel electrophoretic characterizations of isolated t-PA were carried out using a Phast System with silver staining detection (Pharmacia Biotech, Uppsala, Sweden). Polyacrylamide gels (12.5%; Phast Gel Homogeneous 12.5) from Amersham–Pharmacia Biotech were used in the SDS–PAGE method.

The quantity of t-PA isolated from CHO supernatant was established using the enzyme-linked immunosorbent assay (Testkit Imulyse t-PA system, Pharmacia Biopool, Umeå, Sweden). The optical absorption of analyzed solutions was determined at 495 nm using Photometer Immunoreader NJ-2000 (Nunc, Wiesbaden, Germany).

t-PA was isolated from a CHO cell supernatant cultivated in BioPro 1-Medium (Biowhittaker, Verviers, Belgium). The crude supernatant of a fermentation process was treated by centrifugation for 3 min at 1500 rpm using a Labofuge 6000 instrument (Haereus, Germany). The cell-free supernatant was divided into small portions and frozen at −20 °C.

2.3. Methods

2.3.1. Synthesis of poly-L-lysines

Poly-L-lysine was obtained by NCA-polymerization as recently described elsewhere [21].

2.3.2. Solid phase peptide synthesis

All peptides used for affinity binding to t-PA were obtained by solid-phase peptide synthesis using the

tert.-butoxycarbonyl (Boc)-benzyl (Bzl) strategy [22]. In all present cases, the following general procedure for peptide synthesis was explored: (a) DMF, 2×1 mm; (b) DCM, 1×30 mm; (c) ninhydrin test; (d) 20% TosOH/AcOH, 1×60 mm; (f) AcOH, 2×1 mm; (g) DCM, 3×1 mm; (h) DMF, 2×1 mm; (i) 10% TEA/DMF, 1×1 mm; (j) 10% TFA/DMF, 1×2 mm; (k) DMF, 3×1 mm; (1) DMF, Boc-amino acid, 3 equiv, DIC/HOBt, 3 equiv, 1×120 mm.

2.3.3. Immobilization of ligands on CIM epoxy disks

2.3.3.1. Coupling of protein ligands

The CIM epoxy disk was washed with an ethanol, ethanol–water (1:1) mixture and water, then immersed into 0.1 *M* sodium carbonate buffer, pH 9.3, for 2 h and, after that time, was transferred into 1 ml of a 5.0 mg/ml of protein solution in the same buffer. The binding reaction was allowed to proceed over 16 h at 34 °C without any stirring [15]. Then the disk was washed with initial pH 9.3 carbonate buffer to remove the excess unreacted ligand from the porous volume. The carbonate buffer was then replaced by affinity HPMDC mobile phase, i.e., phosphate-buffered saline (PBS, 10 m*M* phosphate buffer containing 150 m*M* of sodium chloride, pH 7.0).

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 the Lowry test [23]. The affinity adsorbents were stored in PBS solution containing 0.02% sodium azide at 4 °C.

2.3.3.2. Immobilization of poly-L-lysine

Similar to the previously described method, the coupling of poly-L-lysine was carried out at static conditions. The 0.1 mol/l sodium borate buffer, pH 10.0, was used as a reactive medium, the concentration of polymeric ligand was 5 mg/ml and the reaction was allowed to proceed over 20 h at 30 °C.

The amount of ligand covalently bound to the support was determined by measuring the decrease in absorbance at 229 nm of ligand solution before and after immobilization, accounting for polymer content in washing buffer volume.

2.3.3.3. Coupling of peptides

The immobilization procedure was carried out according to Ref. [16]. In this case, a washed disk was immersed into 0.1 mol/l 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 20 h without any stirring. Just as in the two previous cases, no quenching of residual epoxy groups of an adsorbent was carried out.

The amount of immobilized peptide was determined according to absorbency at 229 nm of peptide solution before and after the reaction with a sorbent.

2.3.4. Determination of quantitative parameters of dynamic adsorption by HPMDAC

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 [15–17]. For this purpose, model solutions of standard t-PA with concentrations ranging from 0.01 to 0.5 mg/ml were passed through the corresponding disk. Unbound t-PA was removed with PBS buffer and, additionally, the disk was washed with 2 mol/l NaCl. Affinity bound t-PA was eluted with 0.01 mol/l HCl, pH 2.0. The flow-rate at adsorption and desorption was 2 ml/min.

2.3.5. Isolation of t-PA from a crude CHO cell supernatant by affinity HPMDC

One ml of a CHO cell supernatant 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 mol/l sodium phosphate buffer, pH 7.0, containing 2 mol/l NaCl, the adsorbed t-PA was eluted with 0.01 mol/l HCl, pH 2.0. The flow-rate at both adsorption and desorption was 2 ml/min.

2.3.6. Enzyme-linked immunosorbent assay (ELISA)

ELISA was carried out on a polystyrene 96-well microplate according to De Munk et al. [24] to determine t-PA concentration in CHO cell supernatants. For this purpose, the commercially available

ELISA Testkit Imulyse t-PA (Biopool, Umeå, Sweden) was used.

2.3.7. Polyacrylamide gel electrophoresis of t-PA

For SDS–PAGE, lyophilized samples of t-PA were dissolved in a solvent containing 20 m*M* Tris–HCl, pH 8.0, 2 m*M* EDTA, 5.0% SDS, and 0.02% bromphenol blue and treated by boiling for 5 mm 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 SDS–PAGE was done using 0.4% $AgNO_3$. The molecular mass of a sample isolated by HPMDC was established by using electrophoresis low-molecular-mass standard from Pharmacia Fine Chemicals (Uppsala, Sweden).

3. Results and discussion

3.1. Characterization of polymeric and peptide ligands

To establish the molecular mass of poly-L-lysine obtained by NCA-polymerization, SEC and PAGE methods have been used.

In SEC, according to the calibration curve obtained for standard proteins, the apparent molecular mass (more correctly, molecular size) of poly-L-lysine corresponded to 60 000. In contrast to the proteins, the polymer had a high-molecular mass distribution. This conclusion have been done with according the fact that a single broaden peak was observed to be an elution profile. Nevertheless, no additional fractionation of this substance before its use as a specific ligand in affinity chromatography was carried out.

A similar result has been obtained by PAGE method where the proteins of different molecular masses were used as standard markers. The sample of poly-L-lysine was observed as a vertical fuzzy zone with a dense center corresponding to proteins of M_r 60 000.

To determine molecular masses of synthesized linear lysine homologues (K_4, K_8 and K_{12}), SEC has been explored. In this case, carefully characterized peptides of different molecular masses were used as standards. The molecular masses found by SEC analysis appeared to be equal to 1500, 1000 and 550 and, thus, closely corresponded to those of 1554, 1042 and 530 theoretically calculated for K_4, K_8 and K_{12}, respectively. Another useful result to characterize the synthesized lysine derivatives has been obtained by cation-exchange gradient HPMDC. The chromatogram presented in Fig. 1 not only confirms a difference of molecular parameters of peptide products but also allows evaluate their homogeneity (a presence of minor peaks in K_8 and K_{12} samples). This result seems to be very interesting from the point of view of further application of commercially available monolithic supports.

Additionally, the synthesized and chromatographically purified heteropeptides GPRP, K_4GPRP, K_8GPRP, as well as K_{15}A dendrimer were investigated by a standard method of quantitative amino acid analysis. It was found that all prepared peptides had the content corresponded to the theoretically expected one.

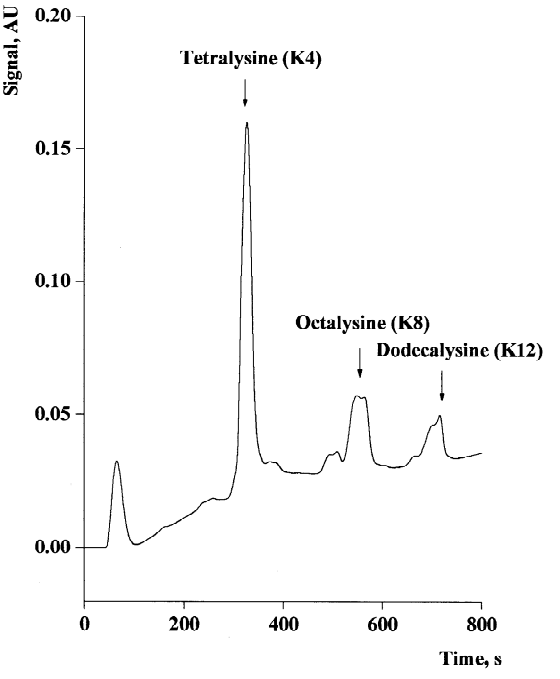

Fig. 1. HPMDC separation of linear lysyl homologues K_4, K_8, and K_{12} on GMA-EDMA SO_3 monolithic sorbent (CIM SO_3 disk). Conditions: 0–1 min gradient from 0 to 50% B, 1–2 min from 50 to 60% B, 2–4 min from 60 to 80% B, flow-rate 5 ml/min; eluent A, 5 m*M* PBS buffer, pH 7; B, 5 m*M* PBS, pH 7 containing 0.5 mol/l NaCl.

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

Ligand	M_r	$q_{immobil}$ [b] (mg/disk)	$q_{immobile}$ (μmol $\times 10^2$/ml)
mAb	150 000	1.2	2.3
Pmg	90 000	1.6	5.5
Fibrinogen	340 000	0.7	0.6
K_n	60 000	1.5	7.2
$K_{15}A$ (dendrimer)	2009[a]	0.8	110.0
K_{12}	1554[a]	0.9	160.0
K_8	1042[a]	0.7	190.0
K_4	530[a]	0.4	200.0
K_8GPRP	1492[a]	0.9	170.0
K_4GPRP	980[a]	0.6	170.0
GPRP	468[a]	0.3	190.0

[a] Molecular masses of peptides were calculated theoretically.
[b] $q_{immobil}$, immobilization capacity of the disks.

All results discussed in this paragraph are summarized in Table 1.

3.2. Immobilization of affinity ligands on glycidyl methacrylate–co-ethylene dimethacrylate (GMA-EDMA) monoliths

Taking into account the very high chemical reactivity of the original epoxy groups of GMA-EDMA macroporous polymers and high concentration of reactive amino groups in the synthetic and natural t-PA ligands studied, the immobilization procedure was carried out as a one-step process at static conditions [15,16]. No intermediate spacers were inserted.

Besides investigation of practical perspectives of synthetic ligands, there was also a fundamental goal to try to compare quantitatively the biospecific binding of t-PA to both synthesized polymers/peptides and its known natural complements such as monoclonal antibodies or the direct substrate plasminogen. Furthermore, it was quite interesting to investigate complementary interactions of the protein of interest not only with its direct biological partners but also with some of their precursors (for example, a precursor of fibrin–fibrinogen).

The results of functionalization of monolithic GMA-EDMA supports by natural and synthetic ligands are presented in Table 1. The data obviously demonstrate a dependence of the resulting concentration of ligand covalently bound to the adsorptive sites (calculated per volume) on molecular mass, more exactly, molecular size, of the functional residue. The molar density of peptide ligands found significantly exceeds that calculated for proteins. The result obtained can be caused, at first, by elevated steric accessibility of intraporous space for small molecules, second, by their higher coefficient of molecular diffusion in comparison with that for large molecules, and, finally, by the absence of the steric blockage of significant surface area following the immobilization of covalently bound protein molecule. In general, the specific concentration of immobilized homo- and heteropeptide ligands seemed to be approximately the same as explained by small differences in their molecular sizes. In contrast, in the case of poly-L-lysine, the molar concentration of immobilized binding sites was significantly decreased. For rigid dendrimer $K_{15}A$ molecules, just as for proteins, a serious decrease of volume concentration of immobilized ligands was observed. This result confirms the structural similarity as well as steric peculiarities of such molecules.

3.3. Study of biospecific pairing of t-PA with different synthetic and natural complements

3.3.1. Affinity adsorption capacity

Frontal elution has been realized for this purpose. It is well known that this experimental approach is sectioned into three stages: (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 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 [15–20], the values of constants of dissociation of biospecific complexes seemed 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 HPMDAC experiments 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. Just such approach has been used in this study.

A schematic scale shown in Fig. 2a, as well as Table 2, summarize the data on affinity adsorption of numerous supports prepared by the immobilization of synthetic and natural t-PA partners on commercial epoxy CIM disks. It is obvious that in the range of ligands investigated, monoclonal anti-t-PA antibodies, mAbs, have been accepted as ''zero-point'' on a scale of measured interactions. For their case, the adsorption capacity was found to be half that predicted from the amount of immobilized ligand. It means that a specific complex with t-PA is formed with participation of every second molecule of mAb covalently bound to the sorbent. This fact can be explained by two factors. First, these are the steric limitations affecting affinity pairing of two macromolecules inside of a rigid porous space. Second, this can be caused by a labiality of conformation of the protein ligand decreasing the accessibility of its active site. It is also impossible to eliminate the effect of surface immobilization of mAb.

The highest adsorption capacity was observed for fibrinogen, an indirect natural partner of t-PA. The ratio $q_{\mathrm{immob}}/q_{\mathrm{ads}}$ shown in the table demonstrates that 25 t-PA molecules interact with one fibrinogen molecule. This may be caused by a less specific adsorption explained by the presence of ''broader'' structure of adsorption site on t-PA molecule bound to fibrinogen. As expected, in the case of using the

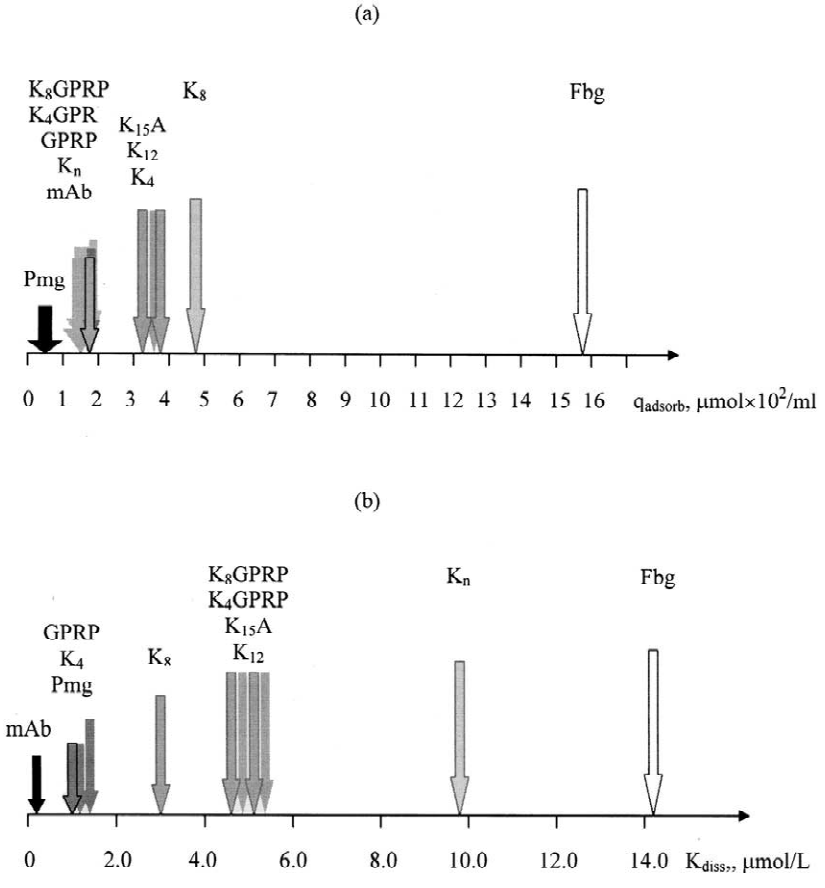

Fig. 2. (a) q_{max} and (b) K_{diss} schematic scales of affinity t-PA–ligand interactions.

Table 2
Affinity characteristics of GMA–EDMA disks

Ligand	q_{adsorb} [a] (mg/disk)	q_{adsorb} (μmol $\times 10^2$/ml)	$q_{immobil}$ [b]/ q_{adsorb}	K_{diss} [c] (μmol/l)
mAb	0.3	1.2	1.91	0.2
Pmg	0.1	0.6	9.17	0.9
Fibrinogen	3.4	15.6	0.04	14.0
K_n	2.4	1.1	6.55	9.8
K_{15}A (dendrimer)	0.9	4.0	27.50	4.9
K_{12}	0.8	3.7	43.24	4.5
K_8	1.1	4.9	38.78	2.7
K_4	0.7	3.4	58.82	1.0
K_8GPRP	0.3	1.2	141.67	5.4
K_4GPRP	0.2	1.1	154.55	4.9
GPRP	0.2	1.1	172.72	1.8

[a] q_{adsorb}, adsorbtion capacity of the disks.
[b] $q_{immobil}$, immobilization capacity of the disks.
[c] Linear regression analysis of the obtained $K_{diss}R^2$ values in the range of 0.92–0.99.

natural t-PA substrate plasminogen as affinity ligand, the interaction seemed to be much more specific.

In comparison with monoclonal antibodies, the homopeptide forms of lysine demonstrated higher values of adsorption capacity. In this case, the ratios q_{immob}/q_{ads} have been found to range from 30 up to 60. That means one t-PA molecule was bound to approximately 30–60 molecules of immobilized peptidyl ligand. Obviously, such a ratio is conditioned by a difference of molecular sizes of interacting molecules. It is easy to imagine that one t-PA molecule forming a specific complex with immobilized ligand blocks a significant amount of neighboring short peptides. At the same time, the obtained values of adsorption capacity practically did not depend on small variations of chain length of the peptidyl lysines. The minor differences in adsorption capacity of homolysine ligands may be due to small experimental differences in volume concentration of ligands discussed. It is also interesting that a transition from linear to branched forms of lysine does not provide any significant change of q_{ads}. The reason may be a conformational variability of lysine ligands caused by the existence of a large number of free ε-NH_2 groups in lysine derivatives. Due to the statistical mode of immobilization, it is not possible to predict precisely whether a single-point or a multipoint linkage with an adsorbent surface will occur, as well as what percentage of free ε-NH_2 groups will be retained in a ligand molecule after its immobilization. In polymeric forms of lysine, a decrease of adsorption capacity has been established. This fact, evidently, can result from the steric hindrance of some adsorption sites at the interaction.

A comparison of q_{ads} for heteropeptide ligands also demonstrates a good coincidence whereas that for lysine hetero- and homopeptides reveals significant difference in this value. The latter has been further explained by the existence of nonspecific ligand–t-PA interactions in the cases of ''pure'' lysyl derivatives.

3.3.2. Dissociation constants

The same frontal elution curves explored for calculation of maximum adsorption capacity have been used to evaluate quantitatively the affinity interactions between t-PA and its immobilized counterparts. The results of analysis of 11 affinity pairs are also shown in Table 2 and Fig. 2b represent, similar to Fig. 2a, a schematic scale of affinity constants.

As expected, the strongest specific interactions were found for standard pair mAb–t-PA. The dissociation constant of this complex seemed to be one order of magnitude lower than those established for

other partners. Unexpectedly, the thermodynamic strength of the enzym–substrate complex [plasminogen (Pmg)–t-PA)] was very close to the immune one. Thus, the obtained data testify to a high degree of preservation of natural structure safety and, accordingly, biological function of proteins immobilized on the surface of GMA-EDMA monoliths. The measured values of K_{diss} confirmed the advantageous parameters of monolithic stationary phases, namely, well controlled structure of the operating surface of the flow-through channels (pores), rapid intrapores mass transport not dependent on molecular diffusion, high concentration of reactive epoxy groups ensuring high surface density of functional ligands covalently bound, etc. At the same time, a thermodynamic stability of a complex of t-PA with its indirect natural partner—fibrinogen—was found to be much lower.

The values of the dissociation constants of a series of synthetic ligands demonstrate high affinity of all of them to t-PA. However, while the values of K_{diss} are of the same order (10^{-6} mol/l), the data analysis of Table 2 enables to identification of the strongest complexes with t-PA as those formed by the short linear peptides GPRP and K_4. Comparing dissociation constants of Table 2, it can be noticed that polymer form of lysine showed less intensive coupling, while the peptide K_{12} and dendrimeric ligand took an intermediate position between polymer and shorter peptides. The obtained results indirectly confirm the increase of probability of multiple-point linkage in the cases of long-chain peptides and polymer macromolecules. In contrast to ''broken'' or ''random'' conformation of immobilized long peptidyl or polymer sequences, the surface conformation of short peptides should be similar to a ''brush''. The data obtained for heteropeptides demonstrate the high similarity of the affinity properties of GPRP-ligand affected neither by introduction of a lysyl residue nor by increasing its length.

Experiments were carried out to exclude any nonspecific interactions using each type of synthesized ligands. For these purposes, the standard mixture of proteins [insulin bovine serum albumin (BSA), lactate dehydrogenase (LDH) and lysozyme] cases, the nonspecific interactions were absent totally, or, in a worse case, did not exceed 2% from applied protein sample.

3.4. Isolation of recombinant t-PA from a crude CHO cell supernatant using affinity HPMDC

All sorbents functionalized with synthetic ligands have tasted for direct isolation of t-PA from a cell supernatant.

Fig. 3 shows an example of t-PA extraction from a crude CHO cell supernatant using disks with immobilized GPRP- and polylysine-ligands. All chromatograms looked very similar and had the same elution times. The exception was a difference in the amounts of nonspecifically bound proteins desorbed with 2 mol/l NaCl. Thus, GPRP-peptide showed a minimum of non-specifically bound protein, whereas a maximum was observed for poly-L-lysine. In fact, all homopeptidyl derivatives of lysine gave higher nonspecific binding in comparison with GPRP and

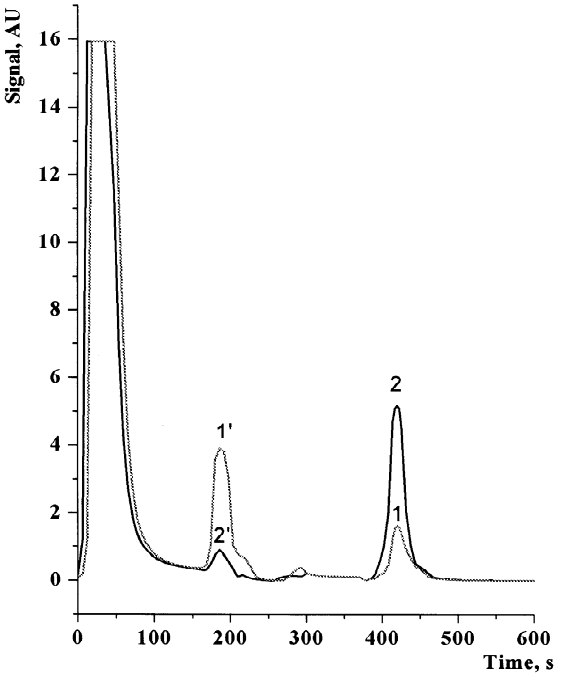

Fig. 3. Affinity HPMDC t-PA isolation from a CHO cell supernatant on epoxy CIM disk with immobilized: (1) K_n (poly-L-lysine), (2) GPRP-peptide. Conditions: flow-rate 2 ml/min; 1 ml of a crude CHO cell supernatant in PBS buffer was loaded on the disk; the disk was washed with 0.01 mol/l sodium phosphate buffer, pH 7.0, containing 2 m*M* sodium chloride; mobile phase of the adsorption step was PBS buffer, pH 7.0; desorption was carried out with 0.01 mol/l HCl, pH 2.0.

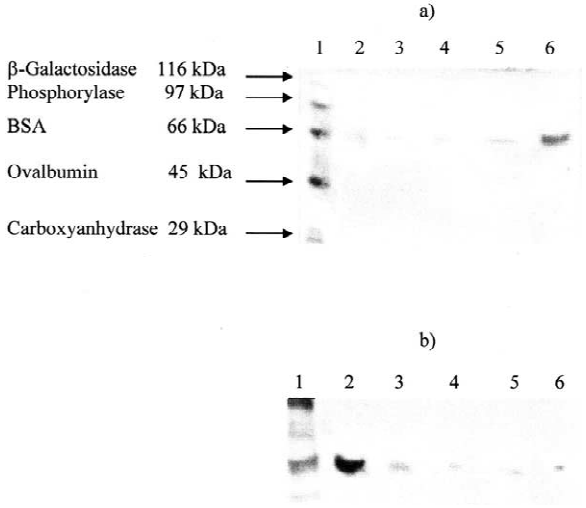

Fig. 4. SDS–PAGE (12.5%) of t-PA isolated from a CHO cell supernatant using affinity HPMDC. (a) Lanes: (1) markers; (6) t-PA-standard; (2–5) t-PA isolated from a supernatant using the disks with the following ligands (lanes): (2) K_n (poly-L-lysine) disk; (3) K_4 disk; (4) K_8 disk; (5) K_8GPRP disk. (b) Lanes: (1) initial proteins from supernatant; (2) t-PA standard; (3–6) t-PA isolated from a supernatant using the disks with following ligands (lanes): (3) K_4GPRP disk; (5) K_{15}A (dendrimer) disk; (6) K_{12} disk.

this increased with increasing length of the homopeptide chain.

The desorbed t-PA was analyzed by SDS–PAGE and ELISA methods. In particular, the purity of isolated product was examined by SDS–PAGE and the results are presented in Fig. 4. The studied synthetic ligands actually were highly specific to t-PA. Another quantitative method, the ELISA test, made it possible to measure the concentration of t-PA both in crude supernatant and eluted samples. According to this method, Table 3 demonstrates that immobilized GPRP and K_{15}A ligands having a good affinity also provided the highest adsorption capacity for t-PA.

Table 3
ELISA quantitation of t-PA isolated from a crude CHO cell supernatant[a] by affinity HPMDC

Synthetic ligands	t-PA (μg)	Yield (%)
Dendrimer K_{15}A	2.5	71
K_{12}	1.3	37
K_4	1.0	29
K_8GPRP	1.4	40
K_4GPRP	1.2	34
GPRP	3.0	86

[a] Total amount of t-PA in 1 ml of supernatant was 3.5 μg.

4. Conclusions

The data obtained in this research show that all the studied lysyl derivatives (homo- and heteropeptides, as well as polymeric forms) obtained by methods of solid-phase peptide synthesis and initiated polycondensation formed highly specific complexes with tissue plasminogen activator (t-PA). The dynamic dissociation constants of affinity pairs determined by frontal elution confirmed the high specificity of all studied ligands.

It has been shown that despite the absence of intermediate spacers between the ligand and the surface of the monolithic medium, the short peptides demonstrated higher affinity to t-PA, whereas increasing the length of the peptide ligand using lysyl polymers led to decreased ligand affinity. This can be explained by a decrease of steric accessibility of adsorption sites of a ligand due to its probable multiple-point binding to a surface.

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 preparative amounts of valuable biological product.

The results of this work have clearly shown for the first time that ultra-short monolithic stationary phases (CIM disks) with well controlled porous design providing enhanced mass transfer are ideal solid-phases for such in vitro investigations of complex biological processes.

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