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Isocratic separations on thin glycidyl methacrylate–ethylenedimethacrylate monoliths

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Abstract

In this work, the isocratic separation of oligonucleotides in the ion-exchange mode on thin glycidylmethacrylate–ethylenedimethacrylate (GMA–EDMA) monoliths in the form of commercially available CIM (Convective Interaction Media) disks is presented. It was found that isocratic separation occurs even on monoliths with a thickness of only 0.75 mm. Peak broadening of the components retained on the monolith is proportional to the retention time, which in turn is proportional to the thickness of the monolith. Peak height is inversely proportional to the retention time. From these results it can be concluded that the mechanism of the separation on such monoliths is similar to that in HPLC columns filled with conventional porous particles. The height equivalent to a theoretical plate of GMA–EDMA monoliths is calculated to be 18.0 μm. The capacity factor k' depends, exponentially, on the salt concentration. The Z factor calculated from fitted equations increases linearly with the oligonucleotide's length. It was also found that the difference between peak retention volume slightly increases with the flow-rate when the experiments are performed in the range from 0.5 to 7 ml/min. From the similarities between the isocratic separations on conventional columns and on thin GMA–EDMA monoliths it is reasonable to believe that separation based on a multiple adsorption/desorption process also occurs in thin monoliths.

Keywords: Glycidyl methacrylate–ethylenedimethacrylate monoliths; Membranes; Monoliths; Stationary phases, LC; Oligonucleotides

1. Introduction

High-performance membrane chromatography (HPMC) is a very effective chromatographic method that combines the advantages of both membrane technology (low pressure drop across a membrane) and column chromatography (high efficiency, capacity and selectivity) [1]. HPMC is based on the separation of target molecules on a very thin separation layer as a result of the interactions between the sample molecules and the active groups on the surface of the pores. Since their introduction, membranes and monoliths have been successfully applied in various chromatographic separations using gradient elution of large biomolecules in extremely short analysis times [2,3]. The main difference between monoliths and conventional HPLC columns lies in the structure of the support. Columns are commonly filled with highly porous particles with a diameter in the range of 3–10 μm. Most of the

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active groups are located within the pores which represent more than 90% of the total accessible surface area and provide a high specific surface area for interactions between molecules in the mobile and stationary phases. For separations under isocratic flow conditions, multiple steps of the adsorption/desorption process should take place. Conventional HPLC columns are, therefore, normally fairly narrow (2–4.6 mm I.D. for most analytical purposes) and rather long (10–25 cm) thus providing a long enough path across the separation layer necessary for the high-resolution separations of different molecules.

In the case of HPMC, the length of the separation layer is much shorter (only up to a few mm). For monoliths made of glycidylmethacrylate (GMA) and ethylenedimethacrylate (EDMA), the maximum thickness of the membrane separation unit (monolith) was defined to be 15 mm [4].

Because of the short separation layer lengths and the resulting short residence times of the molecules within the separation layer, the multiple steps of the adsorption/desorption process were usually not considered as a possible mechanism for the separation. It was even suggested that HPMC using GMA–EDMA monolith membranes does not fall into the category of real chromatography, since this basic chromatographic feature is missing [5]. In fact, the separations of large biomolecules on short columns (membranes) are achieved by selective gradient elution based on the so-called 'on-off' mechanism [6,7]. Only in few cases of some difficult separations of proteins a combination of both 'on-off' and multiple step (differential migration) adsorption/desorption steps were applied [6].

Recently, isocratic separation of plasmid DNA conformers under isocratic flow conditions on a 3 mm thick CIM QA (CIM=Convective Interaction Media, QA=quaternary ammonium active groups) monolithic disk was presented [8]. However, no clear explanation of the phenomena governing the separation mechanism was provided.

In this work, isocratic separation of oligonucleotides in the ion-exchange mode is presented. The effects of the thickness of the separation layer, the mobile phase composition as well as the flow-rate on the separation and peak spreading is discussed in terms of the theory of isocratic separations on conventional HPLC columns.

2. Experimental

2.1. Separation unit

Separation of oligonucleotides was performed on commercially available CIM DEAE (DEAE= diethylaminoethyl groups) disks (BIA Separations, Slovenia). CIM disks are monolithic separation units produced by a radical copolymerisation of glycidyl methacrylate (Aldrich, Steinheim, Germany) and ethylene dimethacrylate (Aldrich) in the presence of pore producing solvents. A detailed polymerisation procedure is described elsewhere [4]. The resulting polymer contains reactive epoxy groups. CIM disks have a diameter of 12 mm and thickness of 3 mm. The DEAE groups were introduced to the CIM epoxy disk by placing the disk into pure diethylamine (Fluka, Buchs, Switzerland) for 24 h at room temperature. The DEAE group density, obtained by a mass difference of the initial and modified disk taking into account the reaction stochiometry, was 0.3–0.4 mmol/disk. In addition, for testing the effect of the length of the separation layer on the separation of oligonucleotides CIM DEAE disks with a thickness of 0.75 mm and 1.5 mm were prepared in the same way.

2.2. Equipment

A gradient HPLC system built with two Pumps 64, an injection valve with a 20 μl SS sample loop, a variable wavelength monitor with a 10 mm optical path set to 260 nm and with a 10 μl volume flow-cell set to response time of 0.15 s, connected by means of 0.25 mm I.D. PEEK (polyether ether ketone) capillary tubes and HPLC hardware/software (data acquisition and control station), all from Knauer (Berlin, Germany) was used in all fast analytical separations. Knauer mixing chamber with its relatively large dead volume was replaced by the PEEK Mixing Tee with an extra low-dead volume (Jour Research, Uppsala, Sweden).

2.3. Sample

The oligonucleotides, synthesised at the National

Institute of Chemistry, Ljubljana, Slovenia, were of the following lengths and structure:

oligodeoxynucleotide 8 (oligo 8): C CAT GTC $T^{3'}$

oligodeoxynucleotide 10 (oligo 10): GTC CAT GTC $T^{3'}$

oligodeoxynucleotide 12 (oligo 12): AG GTC CAT GTC $T^{3'}$

oligodeoxynucleotide 14 (oligo 14): C GAG GTC CAT GTC $T^{3'}$

2.4. Mobile phase

Mobile phase was a 20 m*M* Tris–HCl buffer, pH 8.5 with different concentrations of NaCl.

2.5. Flow monitoring

During the experiments where the influence of the flow-rate on the separation was studied, a validated digital flow meter (K-3773, Phase Separations, UK) was additionally introduced to monitor possible discrepancies between the set and real flow-rates.

3. Results and discussion

To verify the possibility of performing isocratic separations of oligonucleotides on GMA–EDMA monoliths, commercially available CIM DEAE disks were applied. The sample was a mixture of four oligonucleotides with different chain lengths: oligo 8, oligo 10, oligo 12 and oligo 14. The results are presented in Fig. 1 where all oligonucleotides are well separated within 3 min. Their retention times increase with molecular weight. We also observed a pronounced broadening of peaks with increased retention time. To investigate the correlation between peak width and the retention time we performed isocratic runs of a single oligonucleotide oligo 8 as a sample, using mobile phase with different NaCl

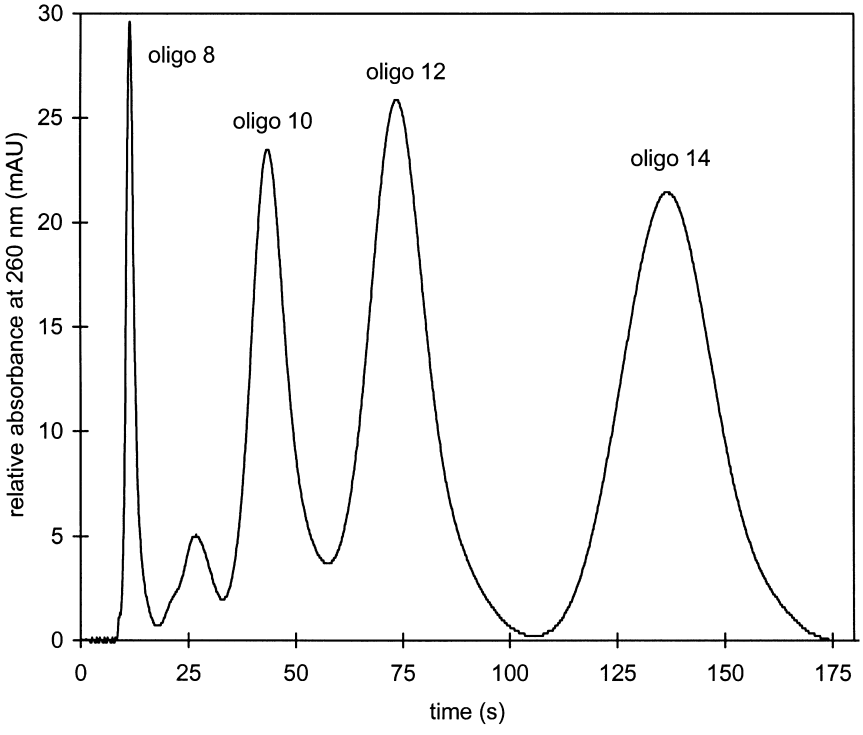

Fig. 1. Isocratic separation of oligonucleotides on a CIM DEAE disk with the thickness of 3 mm. Conditions: Mobile phase: 0.46 *M* NaCl in 20 m*M* Tris–HCl buffer, pH 8.5; Flow rate: 9 ml/min; Sample: 50 μg/ml of oligo 8, 150 μg/ml of oligo 10, 450 μg/ml of oligo 12 and 750 μg/ml of oligo 14 in buffer A; Injection volume: 20 μl; Detection: UV at 260 nm.

concentrations ranging from 1 *M* to 0.38 *M*. The resulting chromatograms are presented in Fig. 2. The increase of salt concentration in the mobile phase decreases the retention time. When 1 *M* NaCl in buffer is used oligo 8 does not bind to the matrix and is eluted with the front. At very low salt concentration (only buffer A) oligo 8 binds irreversibly to the support and is not eluted at all.

According to the theory of isocratic multiple adsorption/desorption separation, the peak width (σ_t) is proportional to the retention time (t_r) [9]:

$$\sigma_t = \sqrt{\frac{\text{HETP}}{L}} \cdot t_r \quad (1)$$

The experimentally determined peak width, σ_t, was measured from the oligo 8 peaks at different molar concentrations of sodium chloride (Fig. 2). The resulting values of σ_t are plotted against the retention time, t_r, in the upper insert in the Fig. 2. The linear relationship between σ_t and t_r as predicted by Eq. (1) is clearly demonstrated ($R^2 = 0.999$). The slope of the line is equal to 0.0774, which translates to the HETP

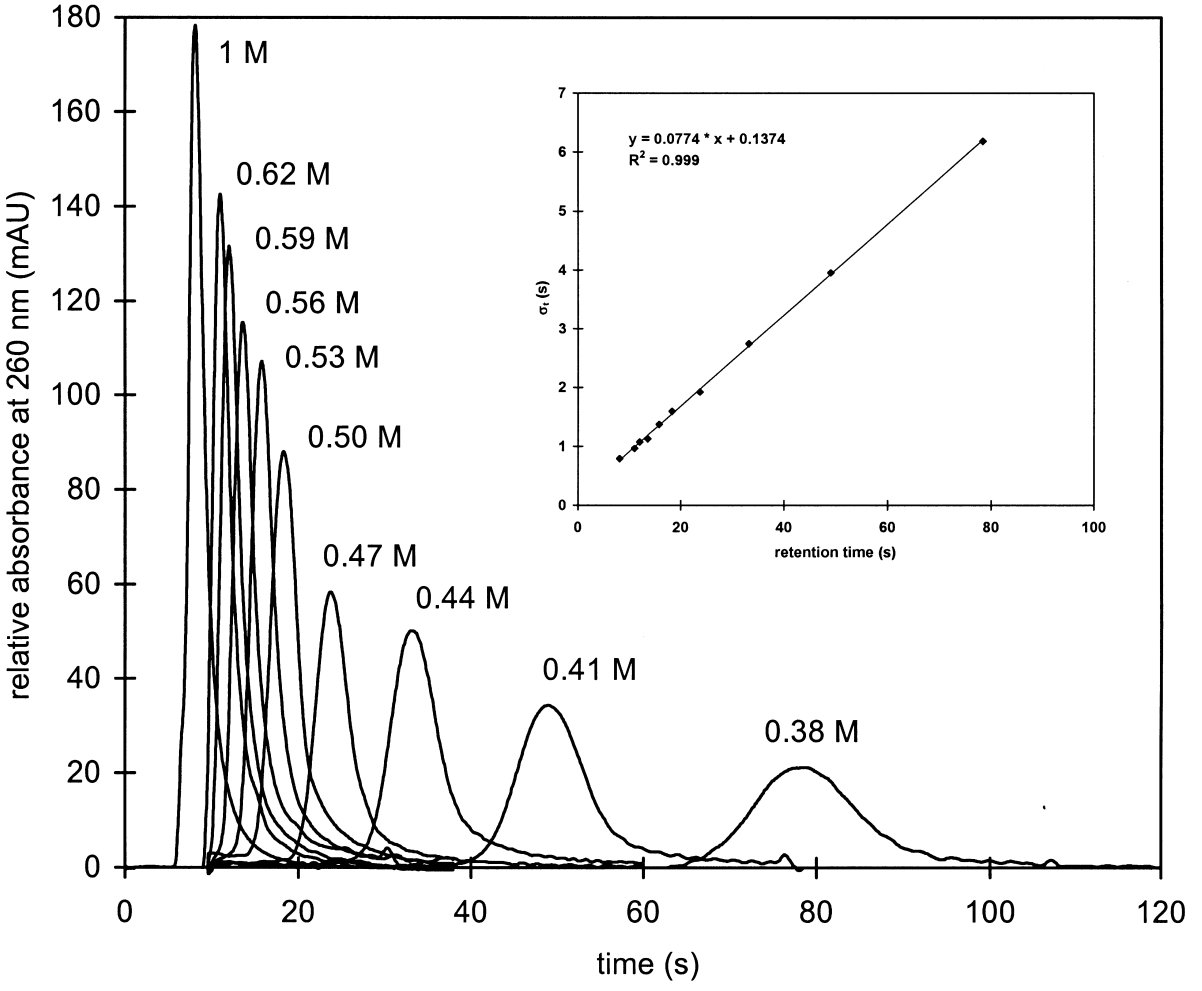

Fig. 2. Effect of the mobile phase composition on the retention time and peak shape of oligo 8 on a 3 mm CIM DEAE disk. Peak width (σ_t) as a function of the retention time is presented in the insert. Conditions: Mobile phase: 20 m*M* Tris–HCl buffer, pH 8.5+different concentrations of NaCl (actual mobile phase composition is shown in the figure); Flow rate: 3 ml/min; Sample: 50 μg/ml of oligo 8 in buffer A; Injection volume: 20 μl; Detection: UV at 260 nm.

(height equivalent to a theoretical plate) value of 18.0 μm. This value approaches the values of HETP for conventional HPLC columns filled with 5–7 μm porous particles.

Furthermore, for the peaks that can be described by a Gaussian function, characteristic for processes based on multiple adsorption/desorption steps, the maximum peak height (h_{max}) is inversely proportional to the retention time [9]:

$$h_{max} = \frac{1}{Rv\sqrt{2\pi \cdot \frac{HETP}{L}}} \cdot \frac{1}{t_r} \quad (2)$$

Experimental values of peak heights of oligo 8 correlated to reciprocal values of the retention times can be successfully fitted with Eq. (2) giving the correlation index $R^2 = 0.988$.

From these results a close similarity between the isocratic separation mechanism on conventional HPLC columns based on multiple adsorption/desorption steps and isocratic separation on the thin GMA–EDMA monolith is indicated.

3.1. Effect of the monolith thickness (column length)

If multiple adsorption/desorption steps occur in thin monoliths, the retention time should be proportional to the monolith thickness [9]. To investigate this, we prepared monoliths of different lengths in the following way: firstly, one disk was placed in the appropriate housing and then the separation of oligonucleotides was carried out. In the next experiment, two disks were placed in the same housing. This progression was continued by placing three and four disks in the same housing. In this way we prepared monoliths with a thickness ranging from 3 to 12 mm which should be equivalent to a single monolith of the same thickness assuming that the interface effects can be neglected. This is justified by similarity between isocratic separation on a single 3 mm disk and a combination of two disks with the thickness of 1.5 mm in the same housing. In addition, we also applied the previously mentioned, specially prepared thin monoliths with a thickness of only 1.5 and 0.75 mm.

Fig. 3 shows that the separation improves significantly with the increase of the column length. In fact, fitting the relationship between the retention time and the column length with the straight line gives correlation index higher than 0.99 for all four oligomeres (see the insert in Fig. 3). Under this salt concentration, separation on the 0.75 mm GMA–EDMA monolith was not achieved. However, after the optimisation of the mobile phase composition, taking the special requirements of the disk related to its thickness into account, the separation of all oligonucleotides was successfully carried out on the ultra thin 0.75 mm GMA–EDMA monolith, see Fig. 4. These data demonstrate that also very thin GMA–EDMA monoliths enable isocratic separation. This rather surprising result could be ascribed to the particular matrix structure and is discussed in details in the last paragraph of the Results and discussion section.

3.2. Effect of the mobile phase composition

To investigate the effect of the mobile phase composition, CIM DEAE disks of thickness 0.75, 1.5 and 3 mm were used. The capacity factor k' at each mobile phase composition was calculated and the results are summarised in Table 1. The capacity factor is correlated to the salt concentration via Eq. [7]:

$$k' = Nc^{-Z} \quad (3)$$

By fitting the logarithm of k' versus the logarithm of the salt concentration, high correlation index was obtained in all cases (Table 1).

These results further support hypothesis that the data obtained by applying CIM DEAE disks can be described by the same equations valid for isocratic elution on conventional columns. Furthermore, by plotting Z values (Table 1) against the length of the oligonucleotide chain, described as a number of nucleotides, a very good linear correlation is obtained (Fig. 5). This makes sense, since with the increase of oligonucleotide length the number of binding sites is also increased and, as a consequence, more salt is needed to solvate the oligonucleotide.

3.3. Effect of the flow-rate

Since monoliths have low diffusion resistance due

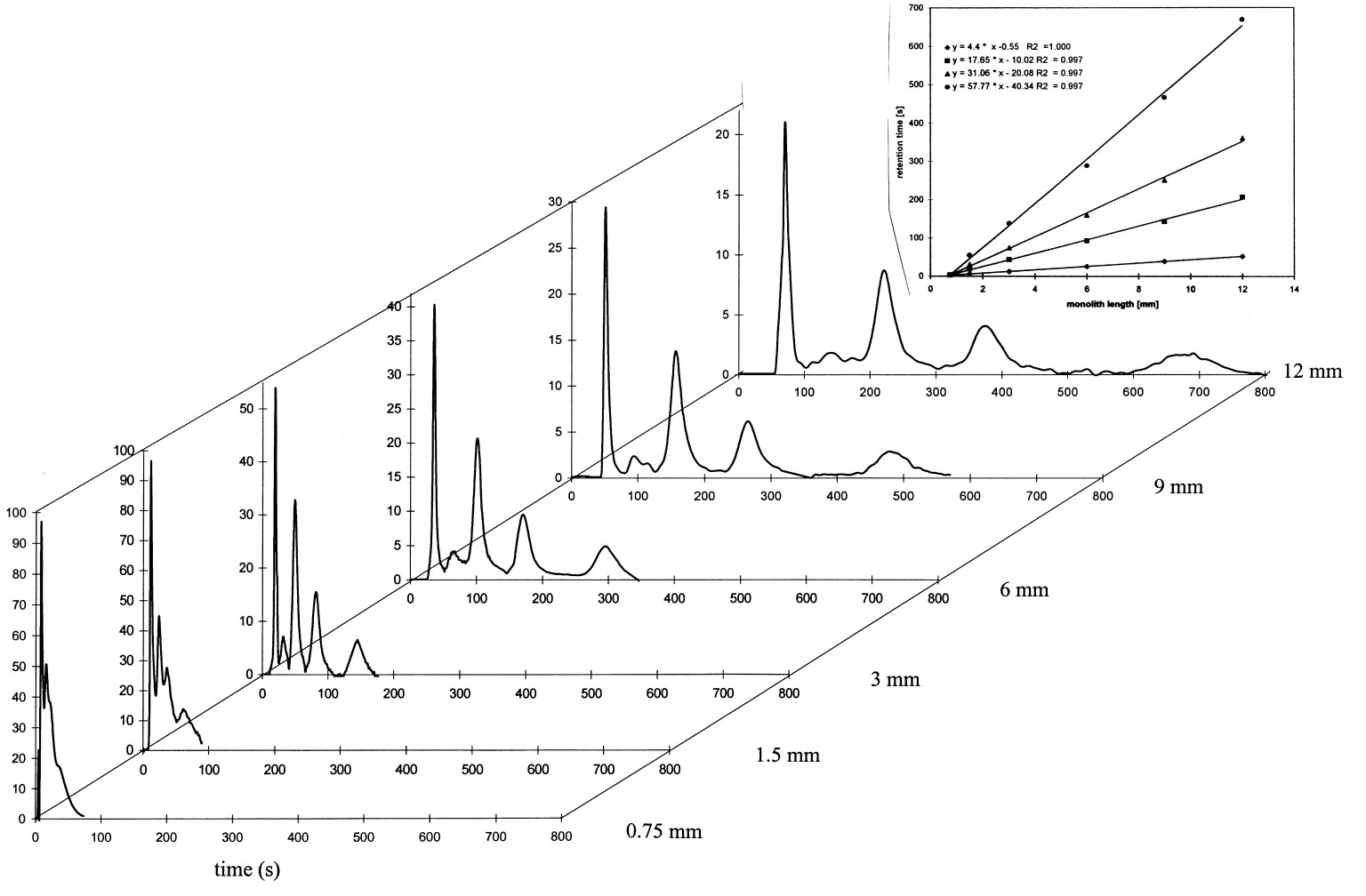

Fig. 3. The effect of the monolith thickness (column length) on the resolution of the oligonucleotide isocratic separation. The retention time dependency on the monolith thickness is presented in the insert. Conditions: Mobile phase: 0.5 *M* NaCl in 20 m*M* Tris–HCl buffer, pH 8.5; Separation unit: one or more CIM DEAE disks in the same housing; Flow rate: 3 ml/min; Sample: 50 μg/ml of oligo 8 (1st peak; ♦ in the insert), oligo 10 (2nd peak; ■ in the insert), oligo 12 (3rd peak; ▲ in the insert) and oligo 14 (4th peak; ● in the insert) in buffer A; Injection volume: 20 μl; Detection: UV at 260 nm.

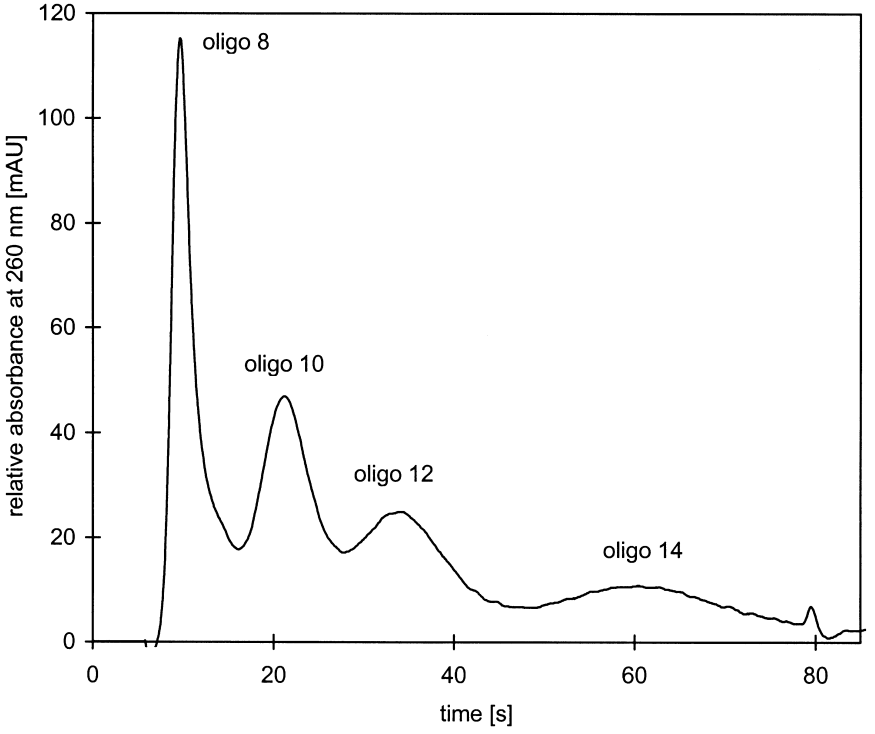

Fig. 4. Isocratic separation of oligonucleotides on a CIM DEAE disk with the thickness of 0.75 mm. Conditions: Mobile phase: 0.37 *M* NaCl in 20 m*M* Tris–HCl buffer, pH 8.5; Flow rate: 3 ml/min; Sample: 50 μg/ml of oligo 8, oligo 10, oligo 12 and oligo 14 in buffer A; Injection volume: 20 μl; Detection: UV at 260 nm.

to convective transport inside the flow-through pores it was shown that the flow-rate has no pronounced effect on the resolution [10]. In contrast to this fact, in the case of isocratic separation of plasmid DNA conformers it was found that there is an optimum flow-rate around 1 ml/min [8]. Therefore, we investigated the effect of the flow-rate on the isocratic separation of four oligonucleotides. Flow rate was

Table 1
Capacity factors k', N, Z and correlation indexes for isocratic separation of oligonucleotides oligo 8, oligo 10, oligo 12 and oligo 14 on CIM DEAE disks of thickness 0.75, 1.5 and 3 mm

0.75 mm					1.5 mm					3 mm				
c_{NaCl} (M)	oligo 8	oligo 10	oligo 12	oligo 14	c_{NaCl} (M)	oligo 8	oligo 10	oligo 12	oligo 14	c_{NaCl} (M)	oligo 8	oligo 10	oligo 12	oligo 14
0.37	2.5	13.2	29.2	67	0.37	4.00	22.4	56.4	145	0.50	2.20	8.77	–	–
0.40	1.5	6.75	13	29	0.40	2.40	10.8	23.2	52.4	0.54	1.34	4.41	6.65	9.79
0.42	1.25	5	8.25	18.25	0.42	1.80	7.80	15.6	33.4	0.55	1.27	4.04	5.63	8.30
0.45	1	3	4.25	–	0.45	1.20	4.40	8	15	0.56	1.18	3.62	4.84	6.63
0.48	0.75	1.75	–	–	0.48	1.00	2.80	4	6.2	0.57	1.10	3.08	4.17	5.41
										0.60	0.79	2	2.35	2.97
$N \cdot 10^2$	2.82	0.66	0.16	0.24	$N \cdot 10^2$	1.69	0.79	0.27	0.11	$N \cdot 10^2$	4.92	3.43	1.57	0.85
Z	4.43	7.63	9.83	10.30	Z	5.43	7.94	9.98	11.86	Z	5.45	7.98	9.85	11.46
R^2	0.978	0.998	0.999	0.999	R^2	0.983	0.997	0.998	0.997	R^2	0.989	0.996	0.995	0.999

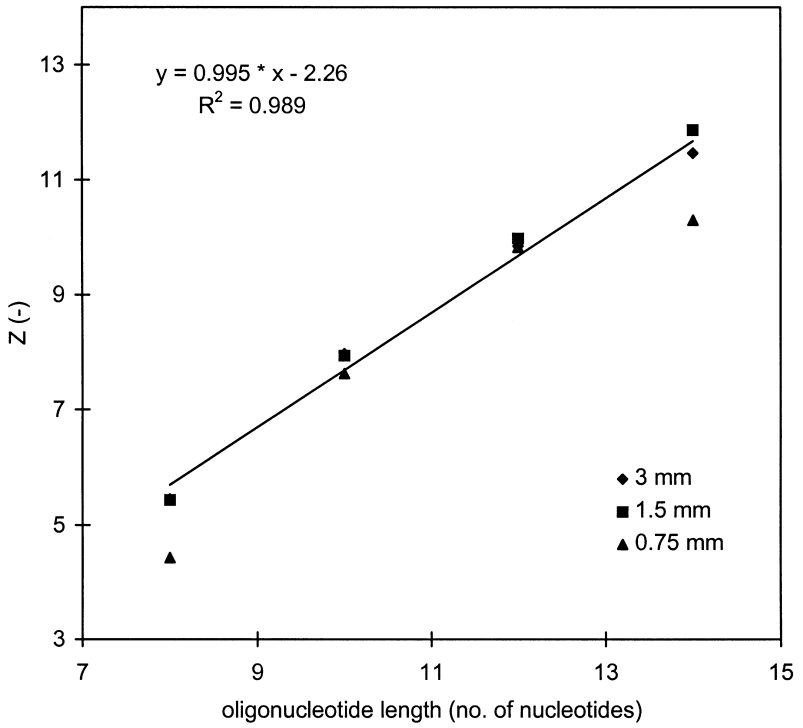

Fig. 5. Effect of the oligonucleotide length on the *Z* factor. Data from Table 1.

measured by a validated flow meter to provide accurate data for further analysis where the results are normalised to the volume of mobile phase run through the GMA–EDMA monolith. The effect of the flow-rate was investigated on CIM DEAE disk of 0.75, 1.5 and 3 mm thickness. Surprisingly, it was found that an increase in flow-rate has a beneficial effect in terms of the difference between peak retention volume. This phenomenon was mostly pronounced on a 3 mm CIM disk (see Fig. 6) and can possibly be explained from the hydrodynamic point of view. Higher flow-rate causes higher back pressure drop on the monolith. As a consequence, mobile phase containing the molecules to be separated can penetrate into pores of smaller diameter [7,8]. In this way, the active surface available for the adsorption/desorption process is larger, possibly resulting in longer retention times.

From these results, another confirmation of the separation process based on multiple adsorption/desorption mechanisms can be derived. During the preliminary experiments, we considered the possibility that the isocratic separation is due to shear forces. In contrast to separations carried out in conventional HPLC columns, where fluid in the pores is stagnant, in monoliths there is a laminar convective flow throughout the pores. This flow causes shear forces on the surface where the molecules are adsorbed. It is possible that these forces could cause some kind of erosion of adsorbed molecules from the surface. Since the molecules are bound to the surface to a different degree because of a varying number of binding sites, they can be selectively desorbed. This mechanism would lead to two effects: since the shear forces increase with increased flow-rate, it is reasonable to speculate that the resolution would decrease. On the other hand, at very low flow-rates shear forces would be probably too low to desorb some strongly bound molecules. However, these two effects were not observed either at a very low flow-rate where also all molecules desorb from the matrix, or at higher flow-rates where the separation even improves and no reduction in retention volume is observed (see Fig. 6). Since both

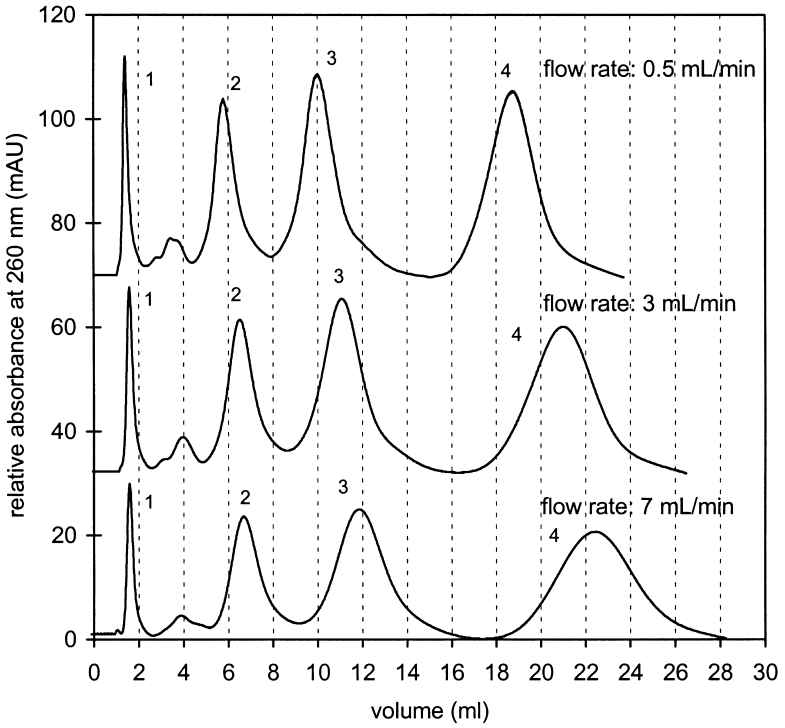

Fig. 6. Effect of the flow-rate on the isocratic separation of oligonucleotides on a CIM DEAE disk with thickness of 3 mm. Conditions: Mobile phase: 0.46 *M* NaCl in 20 m*M* Tris–HCl buffer, pH 8.5; Sample: (1) 50 μg/ml of oligo 8, (2) 150 μg/ml of oligo10, (3) 450 μg/ml of oligo12 and (4) 750 μg/ml of oligo 14 in buffer A; Injection volume: 20 μl; Detection: UV at 260 nm.

effects are different from the expected one in the case of the shear rate elution, the multiple adsorption/desorption process is the most probable phenomenon occurring.

All data show that equations derived for isocratic separations based on multiple adsorption/desorption steps can also be efficiently applied for isocratic separations on thin monoliths. Therefore, it is reasonably to assume that similar mechanisms also occur in these monoliths. It is surprising however, that the 0.75 mm thick monolith can provide sufficient travel length for this phenomenon to occur. There are two characteristics of the monoliths that might help to explain these results. The flow-through pores in the GMA–EDMA monoliths have a broad size distribution [11]. In addition, they are highly interconnected. Therefore, the molecules travelling through the monolith have to pass many constrictions and tortuosities. As a consequence, the real path through the monolith is longer then its physical thickness. Thus, the molecules have more time to interact with the surface. The second characteristic of the monoliths is the reduced diffusion resistance. In the case of particle supports the diffusion through the stagnant liquid inside the pores is a major bottleneck of the mass transfer exchange between the mobile phase and the surface of the support where the active groups are located. In the case of monoliths, this stagnant layer is extremely reduced and the mass exchange is much faster. The main result is that on the same length of the separation layer, many more adsorption/desorption steps can take place in monoliths than in the conventional porous supports. Thus, a much thinner monolith is sufficient for achieving the same number of adsorption/desorption steps. In addition, by comparing the separation of plasmid DNA conformers [8] and oligonucleotides, better separation in the case of the latter was achieved. This might be due to the smaller molecular size of the oligonucleotides and, consequently, faster diffusion.

It can be concluded that, in contrast to the selective gradient elution where the separation resolution commonly improves with increased molecular mass [7], in the case of isocratic separations smaller molecules can be separated more easily.

4. Conclusions

Our results show that is possible to isocratically separate oligonucleotides on anion-exchange CIM DEAE disks and that the performance of the monolith stationary phase is similar to those of 5–7 μm porous particles. From the behaviour of the isocratic separations it was concluded that the mechanism of the separation on thin GMA–EDMA monoliths is very similar to the one on conventional columns.

All these observations indicate that isocratic separation on thin GMA–EDMA monoliths is very probably the result of the multistep adsorption/desorption process. The fact that isocratic separation on the monolith with thickness of less than 1 mm occurs, may be ascribed to convective mass transport through the flow-through pores, which cause an increase in the number of adsorption/desorption steps on shorter monolith lengths. This phenomenon opens many new areas of fast separations or purification on this type of supports. For example, by comparing separation of plasmid DNA conformers and oligonucleotides, much better separation in the latter case was achieved. This is probably due to the lower molecular mass of oligonucleotides and, consequently, higher diffusion coefficients. According to this analogy one can speculate also the possibility of efficient isocratic separation of peptides. Furthermore, it is reasonable to assume, that also other smaller molecules having even higher diffusivities, might also be separated isocratically.

5. Symbols

σ_t	peak width (s)
c	concentration of salt NaCl (mol/L)
h_{max}	maximum peak height (mAU)
k'	capacity factor
L	separation unit length (m)
N	constant
R	probability for the molecule to be in the mobile phase
t_r	retention time (s)
v	linear velocity of the mobile phase (m/s)
Z	stoichiometric parameter related to the number of the moles of the salt needed for the solvation

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References

[1] T.B. Tennikova, F. Švec, J. Chromatogr. 646 (1993) 279.
[2] A. Štrancar, P. Koselj, H. Schwinn, Dj. Josic, Anal. Chem. 68 (1996) 3483.
[3] O.-W. Reif, R. Freitag, J. Chromatogr. A 654 (1993) 29.
[4] F. Švec, T. Bleha, T.B. Tennikova, B.G. Belenki, US Pat., 4 889 632 (1989).
[5] J.M.J. Frechet, F. Švec, US Pat., 5 334 310 (1994).
[6] J.L. Coffman, D.K. Roper, E.N. Lightfoot, Bioseparation 4 (1994) 183.
[7] T.B. Tennikova, R. Freitag, in: H.Y. Aboul-Enein (Editor), Analytical and Preparative Separation Methods of Biomolecules, Marcel Dekker, New York, in press.
[8] R. Giovannini, R. Freitag, T.B. Tennikova, Anal. Chem. 70 (1998) 3349.
[9] J.C. Giddings, Dynamics of Chromatography, Part I, Marcel Dekker, New York, 1965.
[10] A. Štrancar, M. Barut, A. Podgornik, A. Buchaher, Dj. Josic, LC·GC 11 (1998) 660.
[11] A. Podgornik, Ph.D. Thesis, University of Ljubljana, Ljubljana, 1998.