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Journal of Chromatography A, 1065 (2005) 75–81
JOURNAL OF CHROMATOGRAPHY A
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Chromatographic investigation of macromolecular affinity interactions

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Available online 8 December 2004

Abstract

High-performance monolithic disk affinity chromatography was applied to the investigation of formation of complexes between (1) complementary polyriboadenylic and polyribouridylic acids, e.g. poly(A) and poly(U), respectively, (2) poly(A) and synthetic polycation poly(allylamine), pAA. Polyriboadenylic acid and poly(allylamine) were immobilized on macroporous disks (CIM disks). Quantitative parameters of affinity interactions between macromolecules were established using frontal analysis at different flow rates.

Keywords: Affinity chromatography; Monolithic disks; Immobilization; Polyribonucleotides; Polymers; Hybridization

1. Introduction

The use of complementary hybridization of DNA or RNA with their analogues immobilized on solid supports has become a common technique in molecular biology for detection, isolation and genetic analysis of specific sequences [1–3]. Besides, in the last few years the synthetic polycations (homo- or copolymers) began to be actively used in gene therapy as protecting agents or non-viral vectors for gene delivery [4–7]. The formation of polycation–DNA complexes are particularly attractive way for such purposes. To act, they have to be attached to the target cell surface, to be internalised, to leave the endosomes, to find a way to the nucleus, and, finally, to be available for transcription [8]. At the moment, many methods exist to study in vitro the formation of described complexes between DNA and cationic carriers [9,10]. Thus, the interaction of poly(allylamine) with DNA has been studied by such spectral methods as IR, CD, UV and fluororescence spectroscopy [11,12]. The authors have detected the simple electrostatic binding of polycations to DNA via both phosphate groups and nitrogenous bases of DNA macromolecule. The dissociation of such complexes, both in vitro and in vivo, is a crucial point. If the affinity between DNA and polymeric carrier is too low, the complex will dissociate too quickly to act as transport unit, while too strong binding might prevent the necessary intracellular release of DNA. The multiple binding sites on oppositely charged macromolecules result in the integrated stabilization. The cooperative effect of ionic bonds between the cationic polymers and anionic DNA has to be taken into consideration [13–15].

To investigate the noticed complexes we suggest to use affinity chromatography on short monolithic columns (CIM disks). In this case, one of the partners is immobilized on the surface of monolithic support while another one is in the mobile phase. Such approach based on biological specificity has first been published at the end of the 1960s [16,17]. Later, different solid supports such as agarose, cellulose, dextrane, glass, ceramics, silicon wafer, magnetic beads, nylon have been used for the immobilization of nucleic acids [18–24]. However, all these solid phases have significant disadvantages. For example, polysaccharide supports suffer from the deterioration of solvent passage through the column and the destruction of the carrier by microorganisms. For silica sorbents, a gradual loss of immobilized ligands caused by leaching of carrier surface presents a serious problem [25].

The recently developed new type of bioseparation on specially designed macroporous polymeric disks based on poly(glycidyl methacrylate–co-ethylene dimethacrylate)

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doi:10.1016/j.chroma.2004.10.073

(GMA–EDMA) is widely used in different practical fields [26–28]. Originally epoxy bearing GMA–EDMA polymer can be easily transformed into hydroxy, carboxy, sulfo or amino derivatives to realize different chromatographic mechanisms. These disks were recently used as highly selective affinity sorbents as well as the high throughput bioreactor supports [29–35]. Most importantly, the improved mass transfer mechanism allows consideration only the biospecific pairing as a time limiting step. The last fact seemed to be used effectively not only in affinity separation processes but also at in vitro modelling of biological events following the formation of complementary functional pairs [35].

In this paper, the results of use of affinity chromatography on short monolithic columns to characterize the complexation between complementary polyribonucleotides, as well as anionic polyribonucleotide and synthetic polycation are presented. The data obtained can help to make the right choice for development of fast and efficient analytical and preparative methods for nucleic acids purification.

2. Experimental

2.1. Materials and chemicals

The macroporous GMA–EDMA monoliths (CIM disk, BIA Separation, Ljubliana, Slovenia) were used as a stationary phase. The macroporous disks had following parameters: diameter of 12 mm, thickness of 3 mm, volume of 0.34 cm^3, dry mass of 0.34 g, porosity of 70%, mean pore radius of 0.7 μm, specific surface of 10 m^2/g, and initial concentration of epoxy groups 3–5 × 10^{-3} mol/g. The macroporous disks were installed in a cartridge specifically designed by the same producer.

Polyribonucleotides – polyriboadenylic acid [poly(A)] and polyribouridylic acid [poly(U)] (weight-average molecular mass, 200 000–250 000), potassium salt, were from Reanal, Hungary. Poly(allylamine) (pAA) hydrochloride was synthesized as described elsewhere [36]. The M_w of the polymer was found as 8.700.

Double distilled water and analytical grade chemicals purchased from Serva (Heidelberg, Germany) or Sigma (St. Louis, MO, USA) were used to prepare the chromatographic buffers. The obtained solutions were additionally purified by filtration through Milex microfilter (Millipore, USA) with 0.2 μm pore size.

2.2. Instruments

Affinity chromatography was carried out using a system consisting of a peristaltic pump P-1 (Pharmacia, Sweden) and a UV detector (2238 Uvicord S II, LKB, Bromma, Sweden). The chromatographic system was completed with air thermostat (Medical Instrumentation Manufactory, Russia).

The concentration of polyribonucleotides and pAA was determined by measuring of absorbance of their solutions using a UV–vis spectrophotometer SF-26 (LOMO, St. Petersburg, Russia).

2.3. Methods

2.3.1. Immobilization of ligands

Direct covalent procedure was carried out to attach poly(A) and pAA to epoxy groups of disk's material. Besides, two different approach (static and dynamic conditions) were realized to bind poly(A).

2.3.1.1. Direct attachment of poly(A) via NH_2 group of nucleotide base (static conditions). The disk was washed consequently with ethanol, ethanol–water mixture (50:50, v/v) and water, and after that was immersed into 20 mM sodium carbonate buffer (pH 9.3) for 2 h. The covalent attachment of polyribonucleotide was carried out using a single-step reaction between epoxy groups of macroporous polymer and amino groups of adenine of poly(A). For that, the disks were transferred into 1.5 ml of polynucleotide solution in the same buffer of different concentrations ranging from 1.8 × 10^{-3} to 7.6 × 10^{-3} M. The binding reaction was allowed to proceed at room temperature for 24–60 h without any stirring. After the reaction has been finished, the disk was washed with initial carbonate buffer, pH 9.3, to remove the excess of unreacted poly(A) from the sorbent porous volume, then with water, and immersed into 10 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl.

The amount of nucleic acid bound to the support was determined by monitoring the decrease in absorbance of poly(A) before and after immobilization. The concentration of polynucleotides in eluates was calculated using known molar absorption coefficient at maximum wavelength 260 nm [37].

The disk with immobilized macromolecular ligand was stored at 4 °C in 10 mM phosphate buffer containing 100 mM of sodium chloride, pH 7.4, with addition of 0.02% sodium azide.

2.3.1.2. Attachment of poly(A) via NH_2 group of nucleotide base (hydraulic approach). The washed epoxy disk was immersed into 20 mM sodium carbonate buffer (pH 9.3) for 2 h. One millilitre of poly(A) solution with concentration of 4.3 × 10^{-3} M in the same buffer was passed through the disk by a syringe and binding reaction took place at 37 °C for 2 h. The same procedure was repeated twice by passing of initial solution of poly(A). After that the disk was washed with initial carbonate buffer, pH 9.3, and water, and immersed into 10 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl.

2.3.1.3. Covalent immobilization of pAA. The washed epoxy disk was immersed into 10 mM sodium borate buffer (pH 10.0) for 2 h. Then the disk was transferred into 1.9 ml of pAA solution with concentration of 5.8 mg/ml in the same

buffer. The attachment was carried out by a single-step reaction analogously to previous described approach (static conditions). The binding reaction was allowed to proceed at 40 °C for 20 h. The amount of ligand coupled to the support was determined by monitoring the decrease in absorbance of pAA solution before and after the reaction. The concentration of polycation in eluate was calculated using established molar absorption coefficient at the wavelength 225 nm. Then, the disk was washed with initial borate buffer, pH 10.0, and water, and immersed into 10 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl.

2.3.2. Hybridization poly(U) with immobilized poly(A)

2.3.2.1. Formation of hybridic pairs between poly(U) and poly(A) at 20 and 37 °C (statics). The hybridization of poly(U) with immobilized poly(A) was realized at 20 and 37 °C using 10 mM sodium phosphate buffer, pH 7.4 with 100 mM NaCl. Poly(A) disk containing 2.6×10^{-6} mol ligand/ml sorbent was immersed into the solution of poly(U) dissolved in the same buffer at concentration 3.9×10^{-4} M and incubated for different time ranging from 1 min to 20 h. After pairing, the disk was washed with the same buffer using a pump to remove the excess of poly(U) from the porous volume. To split the double helix and release poly(U) from the disk, the temperature was raised up to 60 °C and maintained for 10 min. After that the complementary bound poly(U) was eluted pumping the same buffer at 60 °C (a flow rate 1 ml/min).

2.3.2.2. Isotherms of adsorptive binding of poly(U) to immobilized poly(A) (static conditions). The experiments on the hybridization of poly(U) with immobilized poly(A) was carried out using two different concentrations of bound to the disk polyribonucleotide as a ligand, e.g. 1.4 and 2.6×10^{-6} mol/ml disk. 10 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl at 37 °C was used as a liquid phase. To build the isotherms, poly(A) disks were immersed into solutions of poly(U) dissolved in the same buffer at various concentrations ($0.7–12.4 \times 10^{-4}$ M) and incubated for 30 min at 37 °C. After pairing, the disks were washed with the same buffer at 37 °C to remove the excess of poly(U) from porous volume. To release poly(U) from the disk, the temperature was raised up to 60 °C and maintained 10 min, after that complementary bound poly(U) was eluted with the same buffer at 60 °C (pumping at flow rate 1 ml/min).

2.3.2.3. Isotherms of adsorptive binding of poly(U) to immobilized poly(A) (dynamics). To build the isotherms at dynamic conditions, the chromatographic system has been used. The hybridization of poly(U) with poly(A) was realized using 10 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl at 37 °C as a mobile phase and flow rate 1.0 and 2.0 ml/min was applied in this experimental series. The solutions of poly(U) with different concentrations ranging from 0.8 to 9.5×10^{-4} M were passed through the disk until no further increase in absorption density at the exit of a chromatographic device has been monitored. After hybridization, unbound poly(U) was removed from the pores by washing with the buffer at 37 °C. To release of complementary bound poly(U) from poly(A) disk, the temperature was raised up to 60 °C and maintained for 10 min (stop-flow regime). Then poly(U) was eluted at 60 °C using the same buffer (flow rate 1.0 ml/min).

2.3.3. Isotherms of adsorptive binding of poly(A) to immobilized pAA (dynamic conditions)

Formation of pAA–poly(A) complex was studied using 10 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl, room temperature and flow rate 1.0 ml/min. In this case, the solutions of poly(A) of different concentrations ranging from 0.9 to 8.5×10^{-4} M were passed through the pAA disk until no further increase in the absorption density at the exit of a chromatographic device has been monitored. After that the unbound excess of poly(A) was removed from the pores by washing with the same buffer. The affinity bound poly(A) was eluted using 10^{-4} M sodium hydroxide solution, pH 10.

2.3.4. Determination of parameters of affinity pairing

The affinity characteristics of the poly(A)–poly(U) and pAA–poly(A) pairs such as maximum binding capacity (q_{max}) and dissociation constant (K_{diss}), were evaluated on the basis of mathematical treatment of experimental adsorption isotherms [30,31].

3. Results and discussion

3.1. Covalent attachment of polymeric ligands to GMA–EDMA supports

3.1.1. Immobilization of poly(A)

High reactivity of the epoxy groups of macroporous GMA–EDMA materials together with their high original content ($3–5 \times 10^{-3}$ mol/g sorbent) and porous channel-like morphology allows carrying out covalent binding of any amino bearing ligand as a single step under mild conditions. For the case of used polyribonucleotide, the amino groups of adenine base in 6th position will also be able to form strong C–N bound reacting with epoxy group of a sorbent [38]. Just similar to protein and peptide ligands [33], no intermediate spacers were inserted in all cases discussed. The reaction scheme is presented in Fig. 1.

The binding of poly(A) used at different concentrations to CIM disks are shown in Table 1. Obviously, the higher concentration of polynucleotide, the bigger total amount of this macromolecular ligand immobilized on monolithic support. The calculated reaction yields appeared to be equal to 17.3–20.5% whereas the amount of bound polyribonucleotide varied from 1.4 to 4.6×10^{-6} mol/ml sorbent.

Kinetic curves built for binding of poly(A) to GMA–EDMA support are shown in Fig. 2. Maximum of coupling

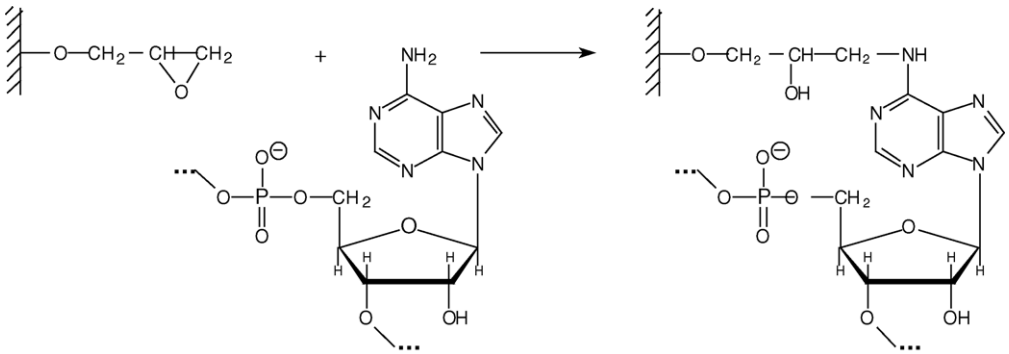

Fig. 1. Scheme of covalent binding of polyribonucleotides to CIM disk.

Table 1
The immobilization of polymer ligands on CIM disk carried out at static conditions

Ligand	Concentration ($\times 10^3$ M)	Immobilized ligand		Reaction yield (%)
		mg/ml disk	mol/ml disk ($\times 10^6$)	
Poly(A)	1.8	0.5	1.4	17.3
Poly(A)	4.4	0.9	2.6	19.9
Poly(A)	7.6	1.6	4.6	20.5
pAA	0.7	6.9	0.8	20.0

was reached in 20 h using the solution with a concentration of of 7.6×10^{-3} M. This result is in a good agreement with that obtained for proteins – another type of macromolecules used as affinity ligands [29]. The most important conclusion is that the inner porous structure of GMA–EDMA disks allows sterically non-limited binding of large molecules differed by their size, conformation and, correspondingly, diffusive properties.

In contrast, the specially developed approach to immobilization based on hydraulic filling of reactive solution into porous space ("syringe pumping") took only 6 h. Though, the calculation of bound to the sorbent ligand is a problem in this case. Indirect information on ligand's capacity can be obtained from the results of affinity adsorption.

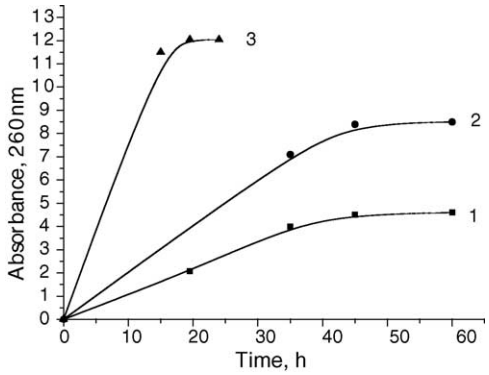

Fig. 2. Kinetic curves of poly(A) binding to CIM disk at different concentrations of polyribonucleotide solutions: (curve 1) 1.8×10^{-3} M; (curve 2) 4.4×10^{-3} M; (curve 3) 7.6×10^{-3} M. The binding conditions were the same as those described under Section 2.3. Aliquots of the reaction mixture were sampled at fixed time and the absorbance at 260 nm was measured.

3.1.2. Immobilization of pAA

Attachment of polycation pAA to CIM disk was carried out at analogous to polyribonucleotide experimental (static) conditions. Calculated yield of reaction was 20% (Table 1).

3.2. Hybridization and release of poly(U)

Hybridization, or the formation of double helix, between dissolved poly(U) and immobilized on the disk poly(A) (see scheme in Fig. 3) was realized also using two, e.g. static and dynamic, experimental approaches. Affinity chromatogram reflecting the hybridization between complementary polyribonucleotides is demonstrated in Fig. 4.

3.2.1. Kinetics of poly(A)–poly(U) hybridization at 20 and 37 °C at static conditions

The kinetic curves of poly(A)–poly(U) hybridization are shown in Fig. 4. Here, poly(A) was bound to the disk and the concentration of macromolecular ligand was equal to 2.6×10^{-6} mol/ml sorbent. Obviously, that affinity coupling of poly(U) to its immobilized complement poly(A) occurs more effectively at 37 °C in comparison with that at 20 °C. In first case, the hybridization was completed in 30 min whereas at 20 °C only approximately 50% of poly(U) applied participated the formation of helix duplex. Thus, all next experiments were carried out using this temperature of hybridization and 30 min as the time needed (Fig. 5).

3.2.2. Determination of affinity parameters

To evaluate both the calculated from experimental isotherms values, e.g. maximum binding capacity, q_{max}, and equilibrium constant of duplex poly(A)–poly(U) dissociation, K_{diss}, which are the important thermodynamic characteristics of all complementary interactions, we used the frontal analysis approach [39]. In this case, a loading was continued until the absorbance of polyribonucleotide solution at the outlet was equal to that at the inlet of the column (disk).

It was established that the obtained adsorption isotherms describing the surface interaction of two macromolecular

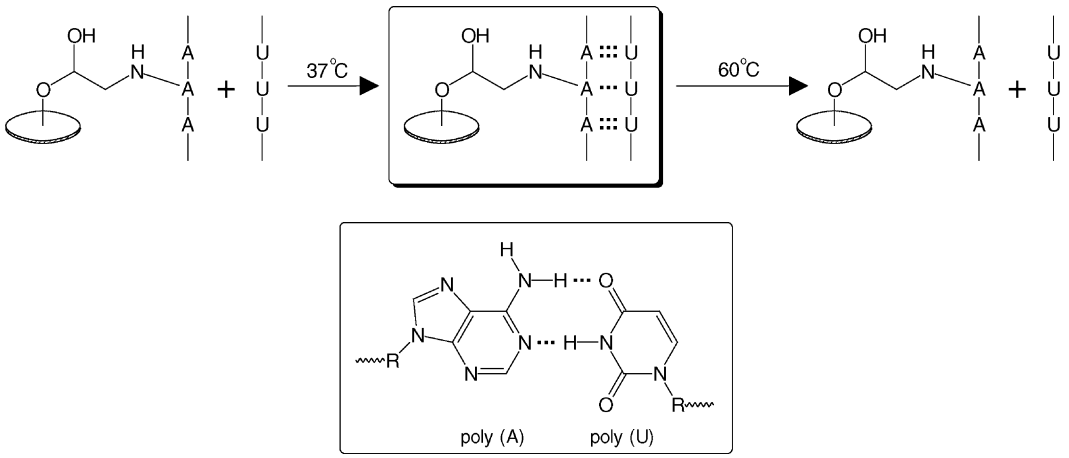

Fig. 3. Scheme of the formation of double helix between dissolved poly(U) and poly(A) immobilized on the disk.

partners fit most often the Langmuir equation [40,41]:

$$q = q_{\max}\frac{C}{(K_{\text{diss}} + C)} \tag{1}$$

where q is the bound polynucleotide in the stationary phase, C is its equilibrium concentration in the mobile phase. Eq. (1) can be rewritten to linearized forms:

$$\frac{C}{q} = \frac{C}{q_{\max}} + \frac{K_{\text{diss}}}{q_{\max}} \tag{2}$$

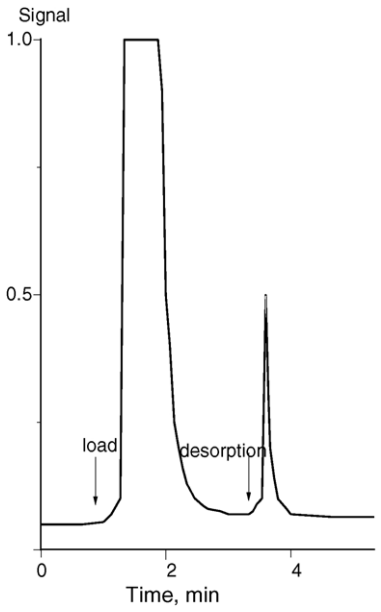

Fig. 4. Affinity chromatogram of the hybridization between complementary polyribonucleotides poly(U) with poly(A) immobilized on CIM disk. Conditions: stationary phase poly(A)-disk (ligand's concentration 2.6×10^{-6} mol/ml sorbent); mobile phase 10 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl; loading 5 ml solution of poly(U) with concentration of 3.8×10^{-4} M at 37 °C; the disk was washed with the same buffer at 37 °C; denaturation with the same buffer at 60 °C; flow-rate 2 ml/min; detection 254 nm.

$$\frac{1}{q} = \frac{K_{\text{diss}}}{q_{\max}C} + \frac{1}{q_{\max}} \tag{3}$$

and both K_{diss} and $q_{\max}$ obtained from the respective plots.

In Table 2 the effect of density (surface concentration) of poly(A), the influence of flow rate and the method of determination of thermodynamic characteristics – static or dynamic – on complementary interaction followed by the formation of polyribonucleotide duplex is demonstrated. It was shown that the increase of ligand concentration did not strongly affect K_{diss} of investigated affinity pair.

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

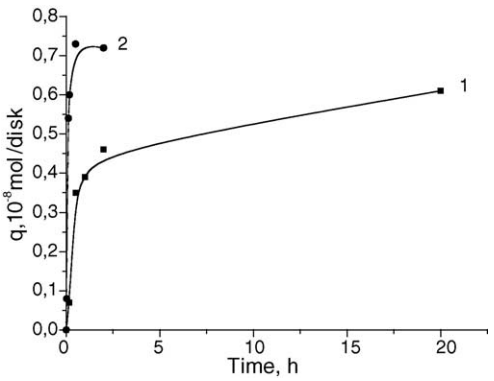

Fig. 5. Kinetic curves binding of poly(U) to poly(A) immobilized on CIM disk (ligand's concentration 2.6×10^{-6} mol/ml sorbent) at temperatures: (curve 1) 20 °C and (curve 2) 37 °C. The concentration of poly(U) was 3.9×10^{-4} M in both cases. The hybridization condition were the same as those described in Section 2.3.

Table 2
Affinity characteristics of the complementary interaction between polyribonucleotides

Experimental mode	Ligand's capacity (mol × 10^6/ml sorbent)	q_{max} (mol × 10^8/ml sorbent)	K_{diss} * ($M \times 10^4$)
Statics	1.4	4.8	4.8 ± 0.4
Statics	2.6	5.3	7.0 ± 0.2
Dynamics, flow-rate 1 ml/min	2.6	3.8	3.4 ± 0.3
Dynamics, flow-rate 2 ml/min	2.6	7.2	3.0 ± 0.4
Dynamics, flow-rate 1 ml/min	4.7	8.7	1.7 ± 0.5
Dynamics, flow-rate 1 ml/min	**	8.2	5.8 ± 0.2

Note: q_{max}: calculated maximum binding capacity of poly(A)-disk; K_{diss}: the equilibrium constant of dissociation of duplex poly(A)–poly(U).

* Linear regression coefficients (R^2) for calculated K_{diss} were found 0.92–0.99.

** Immobilization of poly(A) on CIM disk was carried out at hydrodynamic conditions with concentration of ligand solution 4.3×10^{-3} M.

As it was mentioned above, the calculation of bound to the sorbent poly(A) represents a problem for the case of hydraulic filling of disk's porous volume. According to the quite similar maximum adsorption capacities obtained for both types of affinity disks (see Table 2) using for immobilization the solutions of close ligand's concentration, we can suppose the similar surface concentration of bound macromolecular ligand. Nevertheless, the hydraulic approach is much more attractive being time consuming way to introduce maximum possible amount of adsorption sites.

For a comparison, we studied the binding capacity of poly(A) disk (2.6×10^{-6} mol ligand/ml sorbent) at static and dynamic conditions. We found that the amount of specifically adsorbed poly(U) differed a little (5.3 at statics experimental approach versus 3.8 at dynamics) whereas K_{diss} did not depend significantly on experimental conditions.

The influence of flow rate on affinity binding was studied using the disk with immobilized polyribonucleotide (2.6×10^{-6} mol/ml sorbent), Table 2. For this purpose, binding isotherms were recorded at mobile phase flow rates of 1 and 2 ml/min, respectively, Fig. 6. Under these condition, the affinity constants, K_{diss}, stayed approximately the same, while the maximum adsorption, q_{max}, was increased approximately twice (7.2 and 3.8×10^{-8} mol/ml sorbent at 2 and 1 ml/min, respectively). The difference in results obtained for proteins [31,33] and macromolecules discussed could be probably explained by difference of affinity binding mechanisms. However, to make the right conclusions, the additional experimental work has to be done.

3.3. Determination of parameters of dynamic interaction of poly(A) with immobilized pAA

The same frontal analysis procedure has been used to evaluate quantitatively the affinity interactions between polyanionic and polycationic polymers comparable to the formation of polyribonucleotide duplex. The situation investigated is interesting because of definite difference of polymer structures. Their probable steric compatibility allowing formation of a complex similar to double helix of polynucleotides is still a question.

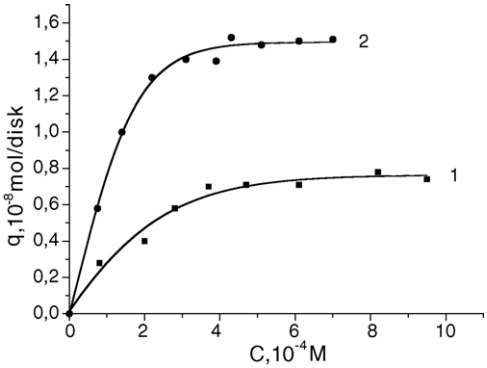

Fig. 6. Comparison of dynamic affinity interactions at different flow rates. Adsorption isotherm obtained for hybridization of poly(U) with poly(A) immobilized on CIM disk (ligand's concentration 2.6×10^{-6} mol/ml sorbent) at increasing concentrations of polyribonucleotide ranging from 0.8 to 9.5×10^{-4} M) at flow rates: (curve 1) 1 ml/min and (curve 2) 2 ml/min. The conditions of affinity pairing were the same as those described under Section 2.3.

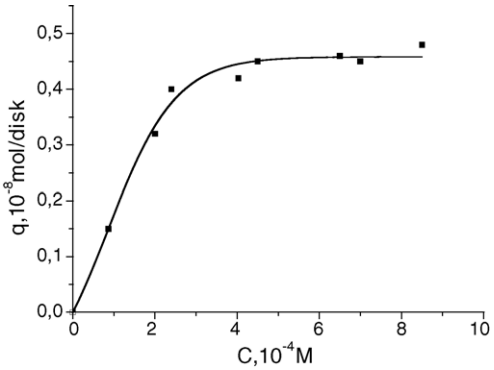

Fig. 7. Adsorption isotherm: hybridization of poly(A) with pAA immobilized on CIM disk (ligand's concentration 1.1×10^{-4} mol/ml sorbent) at increasing concentrations of polyribonucleotide ranging from 0.9 to 8.5×10^{-4} M). The conditions of affinity pairing were the same as those described in Section 2.3.

The adsorption isotherm resulting from frontal experiments and also obeyed to Langmuir's equation is shown in Fig. 7. It has been possible to calculate the dissociation constant which appeared to be quite close to that obtained for polyribonucleotide pair (5.4×10^{-4} M versus 3.4×10^{-4} M).

4. Conclusions

At first time, the experiments on the investigation of specific (affinity) interactions between big complementary polymer molecules have been carried out using macroporous

short monolithic columns (GMA–EDMA disks) as effective stationary phases. It has been shown that affinity mode of HPMDC (high performance monolithic disk chromatography) can be used for quantitative evaluation and comparison of complementary complexes. The method developed and discussed can be further recommended for fast evaluation of DNA- or RNA-binding properties of polymeric candidates planning to be used for gene delivery. Moreover, this approach, after optimization, can be used for fast isolation of chemically or enzymatically synthesized polyribonucleotides from complex reaction mixtures.

Acknowledgements

The authors are grateful to Mrs. I. Gavrilova, IMC RAS, for kindly donated sample of pAA. BIA Separation d. o. o. (Ljubljana, Slovenia) is acknowledged for providing the disks. This project was partly funded by grant of Department of Chemistry and Materials Science, Russian Academy of Sciences.

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