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Study of the specific interaction between L-methionine chromatography support and nucleotides

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ABSTRACT

The interaction of L-methionine-agarose with 5′-mononucleotide was investigated by saturation transfer difference (STD)-nuclear magnetic resonance (NMR) spectroscopy. Chromatographic experiments were also performed using homo-oligonucleotides of distinct molecular masses (1–30 nucleotides) to explore the effect of base hydrophobicity, temperature, pH and salt concentration on the retention of homo-oligonucleotides to L-methionine-agarose support. With STD-NMR, the results reveal that hydrophobic residues, such as the CH_3 of thymine and adenine, can preferentially recognise the L-methionine side chain of the support. Also, 5′-TMP led to more contacts with the support, while 5′-UMP presented fewer STD contacts. For 5′-UMP, 5′-CMP and 5′-GMP, the main interaction with the support was through the sugar-phosphate backbone. Similar binding profiles were obtained using chromatographic experiments. Indeed, 5′-TMP had the highest retention time, followed by 5′-GMP, 3′-AMP, 5′-UMP and 5′-CMP. In general, the retention factor of homo-oligonucleotides was higher for ammonium sulphate concentration 1.5 M. For the $polyT_3$–$polyT_{30}$ series, the retention time increased by about three-fold, indicating that larger homo-oligonucleotides have more hydrophobic bases, thus enhancing contact with the L-methionine support. The temperature (5, 20 and 35 °C) did not influence homo-oligonucleotide retention. However, the retention time slightly increased when the pH was lower than 9. The STD-NMR technique combined with chromatographic experiments was thus successfully used to screen amino acid–nucleotide interactions.

1. Introduction

Understanding the molecular recognition of nucleic acids by proteins is a challenge in structural biology [1]. The recognition of a specific nucleotide sequence is determined by atomic interactions between amino acids and nucleotides [2]. Amino acids have been widely used in biotechnology applications, i.e. as affinity ligands in chromatographic matrices for DNA purification [3]. Indeed, amino acids like histidine, arginine and lysine have been recently used as biospecific ligands in affinity chromatography matrices to purify plasmid DNA from a mixture of impurities [4–6]. Synthetic oligonucleotide sequences have also been applied as models for the interpretation of the interactions that a DNA molecule can promote with an amino acid support, in accordance with the chromatographic conditions [7,8]. L-Arginine and L-lysine supports present similar oligonucleotide retention patterns, in contrast to the L-histidine support [9]. These affinity chromatography data have been explained based on amino acid–nucleotide interaction databases resulting from experimental protein–DNA structures [10,11]. Similar to what has been described for other amino acids, some molecular modelling studies have shown that L-methionine is a hydrophobic, positively charged amino acid that mediates a larger number of contacts in protein–nucleic acid interactions [12]. Also, L-methionine is the amino acid that initiates protein synthesis in all known organisms; it is coupled to a specific initiator, methionine tRNA, by methionyl-tRNA synthetase [13].

Other techniques such as NMR spectroscopy and SPR biosensors have started to be used to identify and characterise polynucleotide binding to amino acid-based supports [14,15]. Taking advantage of this approach, L-methionine-agarose support (see Fig. 1) was selected to study methionine–nucleotide interactions by saturation transfer difference (STD)-NMR spectroscopy. Additionally, chromatographic experiments were employed to study the influence of hydrophobicity, the molecular mass of the homo-oligonucleotides, temperature, pH and buffer composition on the retention of homo-oligonucleotides to L-methionine-agarose.

Our purpose was to establish what kind of interactions are involved between homo-oligonucleotides and the L-methionine support and used these interactions as a model for an interpretation

Abbreviations: NMR, nuclear magnetic resonance; STD, saturation transfer difference; 5′-GMP, guanosine 5′-monophosphate; 3′-AMP, adenosine 3′-monophosphate; 5′-TMP, thymidine 5′-monophosphate; 5′-CMP, cytidine 5′-monophosphate; 5′-UMP, uridine 5′-monophosphate; tRNA, transfer ribonucleic acid.

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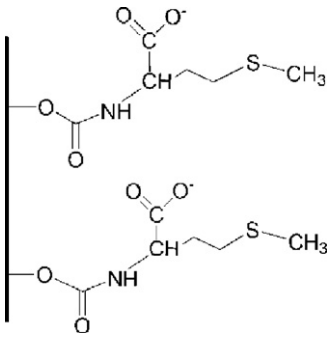

Fig. 1. Schematic structure of the L-methionine-agarose support.

of L-methionine support–nucleic acid interactions. For this purpose, several chromatographic conditions were studied in order to evaluate the specificity and efficiency of affinity interactions.

2. Materials and methods

2.1. Chromatography of oligonucleotides

For the development of chromatographic experiments, analytical-grade ammonium sulphate and Tris(hydroxymethyl) aminomethane (Tris) from Merck (Darmstadt, Germany) were used. The chromatographic experiments were performed in an ÄKTA Purifier system (GE Healthcare Biosciences, Uppsala, Sweden) controlled by UNICORN software, Version 5.11. All the solutions were freshly prepared using ultra-pure grade water, purified with a Milli-Q system from Millipore (Billerica, MA, USA), and the elution buffers were filtered through a 0.20 μm pore membrane (Schleicher Schuell, Dassel, Germany) and degassed ultrasonically. Each class of lyophilised homo-oligonucleotides (polyA, polyT, polyG, polyC and polyU with 1, 3, 6, 15 and 30 bases) was separately dissolved in 10 mM Tris–Cl pH 8.0 and used without further purification. A 10 mm diameter × 20 mm long (about 1.6 mL) column was packed with commercial L-methionine-agarose. This support is characterised by the manufacturer as a cross-linked 4% beaded agarose support with a one-atom spacer and an extent of labelling between 2 and 10 μmol/mL.

To study the influence of ionic strength on the retention of synthetic oligonucleotides used as model molecules, the L-methionine-agarose column was equilibrated at different concentrations of ammonium sulphate (0, 0.5, 1 and 1.5 M) in 10 mM Tris–Cl (pH 8.0) at a flow rate of 1 mL/min. Subsequently, 20 μL (containing 2.5 μg) of each class of homo-oligonucleotides were injected individually onto the L-methionine-agarose column. All the experiments were performed under isocratic elution, and the ionic strength of each analysed sample was corrected according to the ammonium sulphate concentration used during the equilibration step of the respective assay, by dissolving the required quantity of ammonium sulphate in the sample. The absorbance of the eluate was continuously measured at 260 nm. The temperature of each experiment was controlled by connecting the water jacketed column to a circulating Multi-Temp III water bath. A study of the influence of pH (range 7–10) on the retention of homo-oligonucleotides onto the column was conducted by manipulating the pH in the Tris–Cl buffer (10 mM). After each chromatographic run, the column was washed with three column volumes of the buffer without salt in order to remove any molecules that remained bound by hydrophobic interactions.

2.2. STD-NMR spectroscopy

The L-methionine-agarose support and the lyophilised 5′-mononucleotides were purchased from Sigma (St. Louis, MO, USA). The 5′-mononucleotides were added to a sample of L-methionine-agarose in phosphate-buffered saline solution (100 mM K_2HPO_4/KH_2PO_4 and 10% D_2O, pH 8). The final ligand concentration was 68 mM at a 5′-mononucleotide: L-methionine-agarose ratio of 10:1. The ligand resonances were assigned using ^1H–^1H COSY, ^1H–^1H TOCSY and ^1H–^{13}C HSQC NMR spectroscopy.

All NMR experiments were recorded at a temperature of 298 K on a Bruker Avance III 600 MHz spectrometer operating at 14.09 T observing ^1H at 600.13 MHz. The spectrometer was equipped with a four-channel Quadruple (QXI) resonance probe and all spectra were processed with the software topspin 3.0 (Bruker).

The STD-NMR spectra were recorded with 512 scans and selective saturation of L-methionine-agarose resonances at 1.5 ppm (on-resonance) and 30 ppm (off-resonance) using a series EBURP-shaped pulse (50 ms, 1 ms delay between pulses) for a total saturation time of 2.04 s. L-Methionine-agarose resonances are broad and show significant intensity in the region upfield from 4 ppm. Thus, irradiation of 1.5 ppm is expected to result in saturation of L-methionine-agarose resonances and is also considered prudent since the 5′-mononucleotides resonances are present between 8.5 and 3.8 ppm, except for thymidine 5′-monophosphate that has a resonance at 1.85 ppm. In this case, irradiation of L-methionine-agarose resonances was performed at 0.5 ppm. A total relaxation delay of 2 s and eight dummy scans were employed to reduce subtraction artefacts. Subtraction of the on-resonance spectrum from the off-resonance spectrum was performed by phase cycling [16]. The relative STD values were calculated by dividing the STD signal intensities by the intensities of the corresponding signals in a reference spectrum of the same sample $[(I_0 - I_{STD})/I_0]$ [17]. The STD intensity of the largest STD effect was set to 100% as a reference. Reference experiments using the free 5′-mononucleotides themselves were performed under the same experimental conditions to verify true ligand binding. No signal was present in the different spectra, indicating that the effects observed in the presence of the L-methionine-agarose were due to true saturation transfer.

3. Results

3.1. Chromatographic experiments

Chromatographic experiments were performed to study the influence of ionic strength, hydrophobic character of the individual bases of the nucleotides, temperature and pH on the retention mechanism of each class of homo-oligonucleotide (polyA, polyT, polyG, polyC and polyU with 1, 3, 6, 15 and 30 bases), injected individually on the L-methionine-agarose support.

The effect of ionic strength manipulation was evaluated by performing several chromatographic runs using isocratic elution for each oligonucleotide at 20 °C. The ammonium sulphate concentrations tested were 0, 0.5, 1 and 1.5 M in 10 mM Tris–Cl pH 8.0. The results presented in Fig. 2 show that a higher ammonium sulphate concentration promoted an increase in the retention time of each studied homo-oligonucleotide. At a high salt concentration, the homo-oligonucleotide became strongly retained in the column, and could only be eluted by decreasing the salt concentration. This behaviour is probably related to the capacity of ammonium sulphate to induce the displacement of water molecules, which favoured the interaction of homo-oligonucleotides with the L-methionine support.

To evaluate the effect of the hydrophobic character of the individual bases of the nucleotides on retention, some experiments

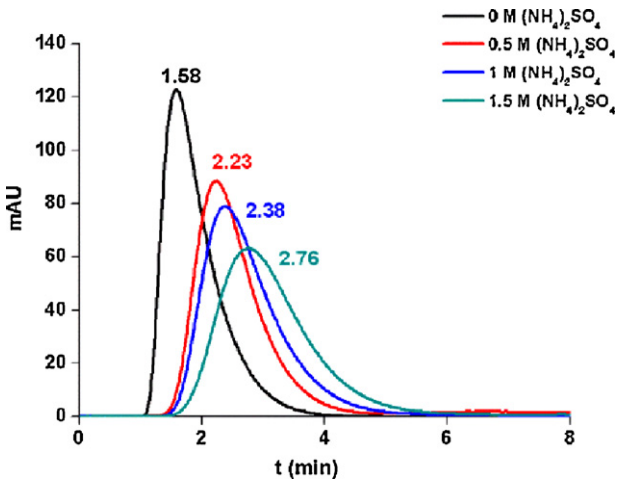

Fig. 2. Overlap of the chromatographic profiles of $polyA_{15}$ homo-oligonucleotide loaded individually and separately onto the L-methionine-agarose support. The curves represent the isocratic elution of $polyA_{15}$ with different $(NH_4)_2SO_4$ concentrations (0, 0.5, 1 and 1.5 M in 10 mM Tris–Cl; pH 8.0) at 1 mL/min and 20 °C.

with mononucleotides (3′-AMP, 5′-GMP, 5′-TMP, 5′-CMP and 5′-UMP) were performed under isocratic elution with the same salt concentration (1.5 M $(NH_4)_2SO_4$ in 10 mM Tris–Cl pH 8.0) at 20 °C. The overall analysis of the results reveals that the magnitude of homo-oligonucleotide retention was mostly dependent on the hydrophobic character [7]. Considering the 5′-mononucleotides, 5′-TMP presented the highest retention time, followed by 5′-GMP, 3′-AMP, 5′-UMP, while 5′-CMP showed the lowest retention time on the L-methionine-agarose support (see Table S1 of supplementary material). This is in accordance with the preference of L-methionine to interact with thymine through H-bonds and water-mediated interactions [11].

Since 5′-TMP was preferentially retained, the interaction behaviour of the following series of homo-oligonucleotides with different sizes $polyT_3$, $polyT_6$, $polyT_{15}$ and $polyT_{30}$ was also evaluated under isocratic elution with the same ammonium sulphate concentration (1.5 M) in 10 mM Tris–Cl pH 8.0 at 20 °C. Fig. 3 presents the overlap of the chromatographic profiles of each thymine homo-oligonucleotide (5′-TMP, $polyT_3$–$polyT_{30}$), which were loaded individually and separately onto the

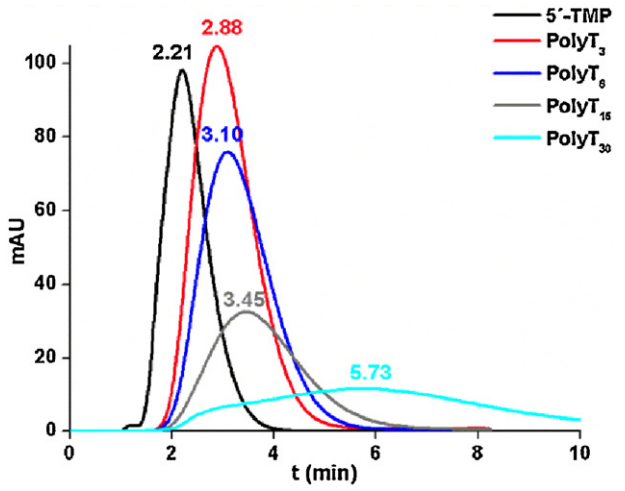

Fig. 3. Overlap of the chromatographic profiles of 5′-TMP, $polyT_3$, $polyT_6$, $polyT_{15}$ and $polyT_{30}$ loaded individually and separately onto the L-methionine-agarose support. Elution was performed at 1 mL/min using 1.5 M of $(NH_4)_2SO_4$ in 10 mM Tris–Cl (pH 8.0) at 20 °C.

L-methionine-agarose column. The results show an increase in homo-oligonucleotide retention time with increased molecular mass. In fact, while maintaining the temperature constant and the salt concentration fixed, the retention time of the homo-oligonucleotides increased with the number of hydrophobic bases of each model molecule. Thus, this behaviour can be related to the number of hydrophobic bases as this enhances the contact points between the larger homo-oligonucleotide and the support.

Additionally, the effect of temperature (5, 20 and 35 °C) was also studied using some homo-oligonucleotides (5′-TMP and $polyT_{15}$) that revealed a preferential interaction with the L-methionine-agarose support while maintaining the ionic strength constant (1.5 M $(NH_4)_2SO_4$ in 10 mM Tris–Cl pH 8.0). The results indicate that temperature manipulation in the studied range did not present a systematic effect on homo-oligonucleotide retention (data not shown). Although the ionic strength used during these chromatographic runs favoured the involvement of hydrophobic interactions, the effect of an increase in temperature did not follow the behaviour of hydrophobic character.

The influence of different pH values (7–10) on the retention of 5′-TMP to the L-methionine-agarose support was also evaluated by performing isocratic elution in the absence (10 mM Tris–Cl) and presence of ammonium sulphate (0.5 M). The results reveal that, in the absence of salt, the retention time was slightly affected by pH variation (see Table S2 of supplementary material). For instance, the retention time of 5′-TMP was slightly higher for lower pH in 10 mM Tris–Cl buffer without ammonium sulphate. However, in the presence of salt (0.5 M $(NH_4)_2SO_4$ in 10 mM Tris–Cl), pH modification did not affect 5′-TMP retention time (see Table S2 of supplementary material). These data suggest that the application of elution buffers without ammonium sulphate and with pH lower than the pKa of L-methionine (9.2) favour and intensify the ionic interactions of this amino acid with homo-oligonucleotides.

3.2. Molecular recognition studies by STD-NMR spectroscopy

One-dimensional STD-NMR spectroscopy was used to accomplish the epitope mapping of 5′-mononucleotides in the L-methionine-agarose binding site. The STD percentages of the 5′-mononucleotides were calculated as the ratio of individual peak intensities in the corresponding difference spectrum and in the reference spectrum and normalised to the highest STD effect.

Fig. 4 presents (a) the ^1H NMR spectrum of 5′-TMP and (b) the STD spectrum of 5′-TMP: L-methionine-agarose while Fig. 5 presents (a) the ^1H NMR spectrum of 5′-CMP and (b) the STD spectrum of 5′-CMP: L-methionine-agarose. The remaining STD spectra of 3′-AMP, 5′-GMP and 5′-UMP are presented in Fig. S1 of supplementary material. Significant STD effects were observed in all spectra collected with and without spin-lock.

The STD spectrum of 5′-TMP is the one with more contacts, suggesting that it is in close contact with the protons of the L-methionine support. The methyl group (100% of saturation) of thymine led to the most prominent STD signal. Moderate signals were observed for $H_{2''}$ (56% of saturation), $H_{2'}$ (55% of saturation) and weaker signals for $H_{4'}$ (16% of saturation) and 5′and 5″ (18% of saturation). These STD percentages show a clear preferential enhancement of the nucleotide base and adjacent parts of the ribose moiety over the phosphate group.

On the contrary, the STD-NMR spectrum of 5′-CMP shows the preference of L-methionine-agarose for phosphate protons $H_{5'}$ and $H_{5''}$ (100% and 97%, respectively). For the ribose moiety, STD effects were only observed for the $H_{1'}$ and $H_{4'}$ protons with intensities of 69% and 71%, respectively. There were apparently no STD responses for cytosine, indicating that this residue is in less intimate contact with the support, corroborating with the chromatographic data presented in Table S1 of the supplementary material.

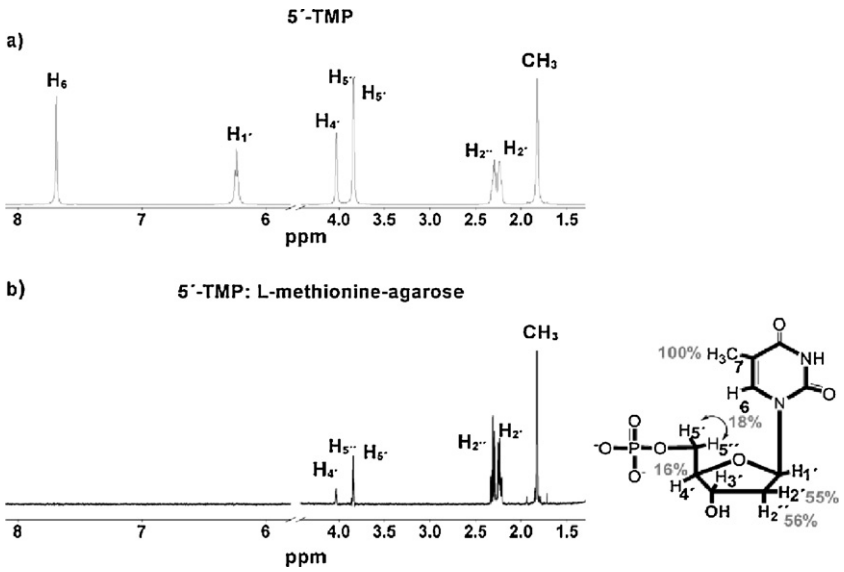

Fig. 4. (a) ^{1}H NMR reference spectrum for free 5′-TMP and (b) STD-NMR spectrum of 5′-TMP: L-methionine-agarose. The ratios of the intensities I_{STD}/I_0 were normalised using the largest STD effect (methyl group) 100% as a reference. Due to the close overlap between the resonances $H_{5'}$ and $H_{5''}$, the integration of the signals was difficult to evaluate and the values for these protons were estimated.

In the case of 3′-AMP, the largest STD effect was observed for adenine proton H_2 (100% of saturation). This suggests that proton H_2 in 3′-AMP might play a predominant role in binding to the support. As occurred previously, significant STD effects were observed for protons at positions 5″ and 5′ (85% of saturation) close to the phosphate group and ribose proton $H_{4'}$ (41% of saturation). No additional STD signals were detected for 3′-AMP.

Binding profiles were similar in the STD-NMR spectrum of 5′-GMP, showing the involvement of the phosphate group in binding to the support due to the highest amount of saturation received by protons at positions 5″ and 5′ (100% of saturation). Also, the adjacent parts of the ribose moiety establish moderate STD contacts with the support through protons $H_{4'}$ (69% of saturation), $H_{3'}$ (42% of saturation) and $H_{1'}$ (23% of saturation). No signal was detected in the STD spectrum of the guanine moiety.

The analysis of the contact map of 5′-UMP reveals that only three protons contributed to binding to L-methionine-agarose, namely phosphate protons $H_{5'}$ and $H_{5''}$ (100% and 93%, respectively) and the proton of the ribose residue $H_{4'}$ (60% of saturation).

On the basis of the data presented here, we can make several statements about the placement of the donor and acceptor atoms involved in the binding site of L-methionine-agarose, even in the absence of their structure. This placement can provide insights into the type of interactions involved.

An overall comparison of the STD-NMR spectra shows that 5′-TMP was the nucleotide that promoted the most STD contacts with the L-methionine support. The main interaction is hydrophobic and involves the methyl group of thymine and probably the side chain of L-methionine. Also, interactions involving sugar C—H might be implicated. The STD data of 3′-AMP showed a base H-bond donor atom possible with the carboxylate group of L-methionine. These results were very similar to those obtained by chromatographic experiments.

The epitope mapping of the remaining 5′-mononucleotides was quite similar; the main interactions might be H-bonds involved in

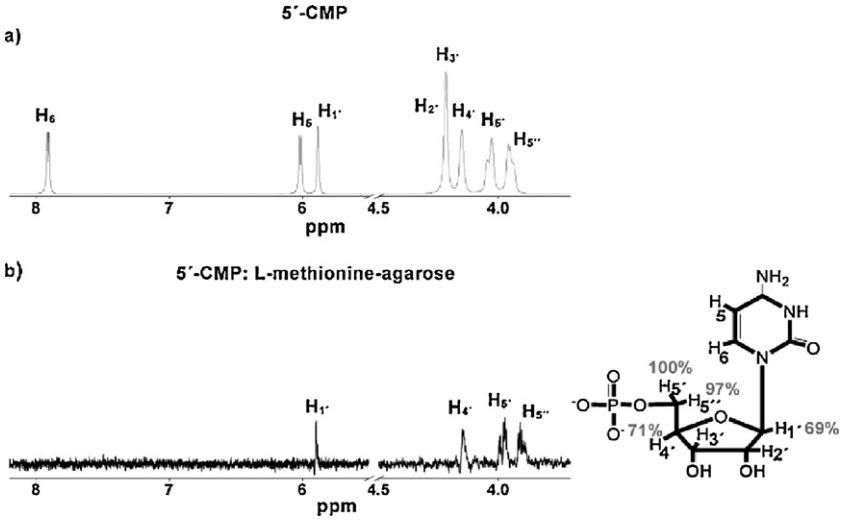

Fig. 5. (a) ^{1}H NMR reference spectrum for free 5′-CMP and (b) STD-NMR spectrum of 5′-CMP: L-methionine-agarose. The ratios of the intensities I_{STD}/I_0 were normalised using the largest STD effect ($H_{5'}$) 100% as a reference.

the sugar donors and $H_{5'}$ and $H_{5''}$ with the sulphur atom of methionine that can be a moderate H-bond acceptor. Moreover, it was shown that L-methionine can do bifurcated H-bonds and act as a double donor type [18].

4. Discussion

To optimise specific binding to L-methionine-agarose, we studied polynucleotide binding using chromatographic and STD-NMR experiments. From the chromatographic analysis, we checked the influence of ionic strength, the hydrophobic character of the individual bases of the nucleotides, temperature and pH on the retention mechanism of each class of the synthetic homo-oligonucleotides on the L-methionine-agarose support. The spectroscopy study identified the most important atom pairs involved in specific nucleotide recognition by the L-methionine support. The homo-oligonucleotides were used as models to understand several interactions with the L-methionine support by changing and optimising the chromatographic conditions.

The chromatographic experiments were initiated by evaluating what kind of salt and ionic strength were needed to promote interactions between the model homo-oligonucleotides and the L-methionine-agarose support. Compared with other previously studied amino acid-supports, L-methionine-agarose promotes an interaction pattern similar to L-histidine-agarose, since the interaction of the homo-oligonucleotides intensified with an increase in the ammonium sulphate concentration [7]. On the contrary, the retention of these model molecules on L-arginine-agarose occurred with a low ionic strength of sodium chloride [8]. Furthermore, the results also show that the interactions established between some homo-oligonucleotides and the L-methionine-agarose support were more intense than those established with L-histidine-agarose when the same ionic strength was applied (ammonium sulphate 1.5 M). These findings suggest that L-methionine-agarose can be an alternative support to L-histidine-agarose in the purification of several biomolecules, such as nucleic acids with a predominance of thymine bases, by taking advantage of the milder elution conditions. Given that the presence of salt favoured the establishment of hydrophobic interactions, the 5′-mononucleotides that showed higher retention under isocratic elution with 1.5 M of ammonium sulphate were 5′-TMP, followed by 5′-GMP, 3′-AMP, 5′-UMP and 5′-CMP. This retention behaviour follows the hydrophobicity of the nucleotides. This result was also confirmed by the STD-NMR data (see Fig. 4) by an increase in the number of STD responses in the case of 5′-TMP. In addition, the individual run of thymine homo-oligonucleotides with different sizes revealed an increased retention time for molecules with a higher molecular mass, as indicated in Fig. 3. Therefore, in the absence of additional effects (salt concentration and temperature variations), the retention time of the homo-oligonucleotides followed the number of hydrophobic bases.

The effect of pH, in the range between 7 and 10, on the retention of 5′-TMP by the L-methionine-agarose support was also evaluated. The retention time increased with a pH lower than the isoelectric point of L-methionine (9.2) and in the absence of ammonium sulphate, suggesting that electrostatic interactions also occur between the phosphate groups of the homo-oligonucleotides and L-methionine, in spite of hydrophobic interactions. Interestingly, when this study was accomplished in the presence of ammonium sulphate (0.5 M), pH manipulation was not reflected in the retention of 5′-mononucleotides, probably because other interactions were favoured under these conditions, such as hydrophobic interactions. Thus, by taking into account the established elution conditions, different interactions can prevail in the binding of homo-oligonucleotides to the L-methionine-agarose support.

The temperature effect (5, 20 and 35 °C) did not influence the retention of the tested homo-oligonucleotides (5′-TMP and $polyT_{15}$) on the L-methionine-agarose support. From the STD-NMR experiments, no significant differences were obtained in the STD signals of 5′-TMP at 5 °C and 35 °C. The results of pH and temperature manipulation in the L-methionine-agarose column were also similar to the previous study with L-histidine-agarose [7].

In conjunction with the chromatographic experiments, the data indeed provide fine details of the molecular recognition of homo-oligonucleotides by the L-methionine-agarose support.

5. Conclusions

STD-NMR spectra combined with chromatographic experiments clearly defined the molecular recognition of homo-oligonucleotides to the L-methionine-agarose support. The STD spectra of the 5′-mononucleotides were quite different; for example, 5′-TMP and 3′-AMP interacted with the support preferentially through the base, whereas for 5′-UMP, 5′-CMP and 5′-GMP, the main interaction was through the sugar-phosphate backbone. The results also indicate that 5′-TMP showed more STD signals, while 5′-UMP showed the lowest STD effects. These findings are similar to the results obtained in chromatographic experiments using 5′-mononucleotides. The polynucleotide retention time was dependent on an increase in the salt concentration and polynucleotide molecular mass. It was also concluded that temperature did not influence homo-oligonucleotide retention. However, the retention time increased slightly when the pH was lower than the pK_a of L-methionine. Hence, our results showed that L-methionine-agarose promote multiple interactions with homo-oligonucleotides, at different experimental conditions, resulting in specificity to recognise nucleotides sequences.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.09.037.

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