

Covalently bonded DNA aptamer chiral stationary phase for the chromatographic resolution of adenosine

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Abstract In this work, a target-specific aptamer chiral stationary phase (CSP) based on the oligonucleotidic selector binding to silica particles through a covalent linkage was developed. An anti-D-adenosine aptamer was coupled, using an in-situ method, by way of an amide bond to macroporous carboxylic acid based silica. Frontal chromatography analysis was performed to evaluate the column properties, i.e., determination of the stationary phase binding capacity and the dissociation constant of the target-immobilized aptamer complex. It was found that such covalent immobilization was able to maintain the aptamer binding properties at a convenient level for an efficient enantioseparation. Subsequently, the separation of adenosine enantiomers was investigated under different operating conditions, including changes in the eluent's ionic strength and the proportion of organic modifiers as well as column temperatures. It was demonstrated that, under various conditions of use and storage, the present CSP was stable over time.

Keywords Chiral stationary phase · Aptamers · Adenosine enantiomers

Introduction

The development of chiral selectors (CSs) specifically designed against the racemate to be resolved (target-specific CSs) has received much attention in the last decade. Target-specific CSs have been mainly designed using molecular

imprinting technology, the production of enantioselective antibodies or a combinatorial strategy. Two categories of combinatorial methods have been described for the generation and the development of specific CSs [1]. The combinatorial strategy from a small library of low molecular weight selectors was the first combinatorial method described. More recently, a very efficient combinatorial approach from a very large library of single-stranded oligonucleotides has allowed the creation of a new class of CSs based on nucleic DNA and RNA aptamers. Nucleic acid aptamers have been successfully used as chiral stationary phases (CSPs) and chiral additives for high performance liquid chromatography (HPLC) or capillary electrophoresis applications [2–8].

At the present time, different aptamer-based CSPs have been developed using specific oligonucleotides bound to the chromatographic particles via a noncovalent biotin–streptavidin bridge [2–6]. Despite the simplicity and the rapidity of such an immobilization method, this kind of binding procedure presents, in terms of reusability and column lifetime, some major drawbacks for practical chromatographic use. Although the streptavidin–biotin link is very strong in solution, the magnitude of the streptavidin–biotin binding constant is greatly reduced when the protein is bound to a surface [9]. In addition, dissociation of the biotin–streptavidin complex can occur at relatively low temperatures, notably in nonionic aqueous solutions or in a medium containing a low salt concentration [10]. Furthermore, as classically observed with proteins, the immobilized streptavidin can be also irreversibly denatured under standard reversed-phase chromatographic conditions, for example, by using a mobile phase containing organic modifiers [11]. So, it appears of great interest to use another kind of oligonucleotide immobilization strategy in order to design more robust aptamer-based CSPs.

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Various immobilization procedures have been described to link covalently macromolecular receptors such as proteins or nucleic acids to silica surfaces [11–16]. Jarrett et al. [15, 16] have notably described a coupling procedure involving the immobilization of ligands containing primary amino groups on macroporous carboxylic acid based silica by way of an amide bond. It was demonstrated that such a technique (1) offers a more efficient and a faster coupling than those involving the use of glycidyoxypropyl-silica and (2) is useful for an in situ coupling procedure [15]. In this paper, we evaluate a target-specific aptamer CSP based on the selector binding to silica particles through this kind of covalent linkage. A 5'-amino-modified anti-D-adenosine DNA aptamer, previously used as a target-specific CS [3], was coupled to carboxylic acid based silica using an in situ method. The resulting CSP was subjected to a frontal analysis in order to characterize the properties of the stationary phase. The effects of various chromatographic parameter changes on the adenosine retention, apparent enantioselectivity and resolution were analyzed. The storage conditions and the column stability over time were also evaluated.

Experimental

Reagents and materials

D-Adenosine was obtained from Sigma Aldrich (Saint-Quentin, France). *N*-Hydroxysuccinimide (NHS) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDAC) were supplied by Accros Organics (Noisy le Grand, France). L-Adenosine was purchased from Chemgenes (Ashland, USA). Na₂HPO₄, NaH₂PO₄, KCl, MgCl₂ and thiourea were supplied by Prolabo (Paris, France). Methanol, ethanol and 2-propanol (HPLC grade) were purchased from Fisher Scientific (Loughborough, UK). HPLC water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse-osmosis cartridge. Macrosphere-WCX carboxylic acid based silica (300-Å pore size, 7-µm particle diameter, 500 µmol of free carboxylate per gram of silica) was supplied by Alltech (Templemars, France). Such carboxylic acid based silica contains an eight-atom chain terminating in a carboxyl group, allowing adequate spacing between coupled ligands and the support surface (Fig. 1a). The 5'-amino-modified DNA oligonucleotide (sequence 5'-ATTATACCTGGGGGAGTATTGCGGAGGAAGGTA TAAT; note, ATTATA and TAATAT sequences were inserted at the 5' and 3' ends of the highly conserved sequence) with a C12 spacer arm (Fig. 1b) was synthesized and HPLC-purified by Bioneer (Buckingham, UK). Unless otherwise stated, the mobile phase used was constituted of

20 mM phosphate buffer, 25 mM KCl, 15 mM MgCl₂ (adjusted to pH 6.0).

Apparatus

The HPLC system consisted of a Shimadzu 10AT liquid chromatograph pump (Sarreguemines, France), a Shimadzu SIL-10AD autoinjector, a Shimadzu SPD-10A UV-vis detector (detection at 260 nm), a Shimadzu SCL-10A system controller with Class-VP software (Shimadzu) and an Igloocil oven (Interchim). The column temperature was fixed at 24 °C, unless otherwise specified.

Column preparation

Firstly, PEEK microbore columns (200 mm×1.0 mm) were packed in-house from a water-methanol (50:50, v/v) slurry of native WCX silica particles, as previously described [3]. Subsequently, synthesis of the CSP was accomplished inside the prepacked column. The previously reported coupling protocol [16] was slightly modified for the aptamer immobilization. Briefly, the column was placed in the chromatograph and washed with water and 2-propanol. For the silica activation, 200 mg NHS and 330 mg EDAC were then applied to the column at a flow rate of 50 µL/min using 2-propanol as the mobile phase. The resulting activated silica column was washed with 2-propanol, methanol, water and phosphate buffer (400 mM, pH 7.2). Prior the coupling procedure, the 5'-amino-modified DNA aptamer was renaturated by heating oligonucleotide at 70 °C for 5 min in the buffer and was left to stand at room temperature for 30 min. The aptameric solution (90 nmol) was then circulated through the column at a low flow rate for about 100 min at room temperature to allow the ligand coupling (Fig. 1c). The column was then washed extensively with buffer to allow the *N*-hydroxysuccinimide esters to hydrolyze back to unreactive support. When not in use,

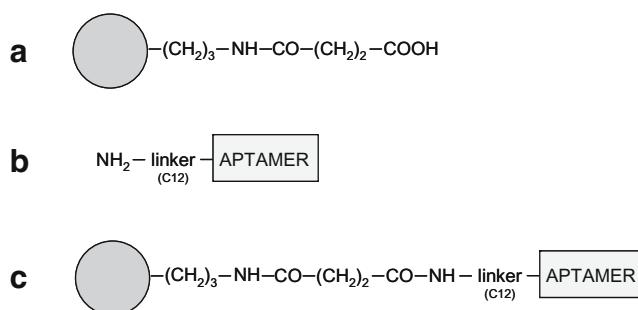


Fig. 1 (a) Macrosphere carboxylic acid based silica, (b) 5'-amino-modified aptamer and (c) DNA aptamer coupled to derivatized silica by an amide bond (activation using *N*-hydroxysuccinimide and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; see the text)

the column was stored in a buffer containing sodium azide (0.05%) or in various buffer–organic solvent mixtures.

Determination of the binding parameters by frontal analysis chromatography

Frontal analysis chromatography was used to quantitatively evaluate column loading and the dissociation constant (K_d) between D-adenosine and the immobilized DNA aptamer. This method is based on the following relationships between K_d and the chromatographic parameters, assuming a langmuirian behavior:

$$(V_R - V_M) = \frac{m_L}{K_d + C}, \quad (1)$$

where m_L is the total amount of active binding sites of aptamer in the column, V_M is the column void volume and V_R is the volume required to elute a continuously applied concentration of D-adenosine (C) from the column. Rearrangement of the above equation gives the following form:

$$\frac{1}{C(V_R - V_M)} = \frac{K_d}{m_L} \frac{1}{C} + \frac{1}{m_L}. \quad (2)$$

According to Eq. 2, a plot of $1/[C(V_R - V_M)]$ versus $1/C$ should yield a straight line with slope corresponding to K_d/m_L and ordinate intercept corresponding to $1/m_L$.

The interaction of D-adenosine with the immobilized aptamer was quantified by subjecting solutions of D-adenosine ($C=5\text{--}50\ \mu\text{M}$) on the column using an aqueous buffer as the mobile phase. The injection system, equipped with a 530- μL sample loop, was switched to the inject position for the entire chromatogram. The experiments were carried out at a flow rate of 50 $\mu\text{L}/\text{min}$, where local equilibrium conditions were established. The first derivative for the advancing elution profile of the plateau was determined to obtain the mean elution volume of the boundary (V_R). V_M was determined similarly using thiourea as a noninteracting solute. Here, it is important to point out that the adenosine enantiomers did not present any significant nonspecific interaction with a control column (of the same dimensions and packed with unmodified silica particles), i.e., solutes were eluted in the void volume.

Determination of the chromatographic parameters

Solute samples were prepared in the mobile phase (0.1 mM of each enantiomer) and injected (100 nL) in triplicate. The mobile phase flow rate was 50 $\mu\text{L}/\text{min}$. The apparent retention factor k was determined using the following relation:

$$k = (t_R - t_0)/t_0, \quad (3)$$

where t_R is the retention time of the enantiomer and t_0 the retention time of the unretained species (thiourea). Although

this is not the most accurate approach for estimating the retention factor, t_R was determined through the apex of the solute peak. This simplification was justified because no thermodynamic or kinetic data were extracted from this chromatographic parameter. The retention times and column void time were corrected for the extra-column void time. They were assessed by injections of solute onto the chromatographic system when no column was present. The apparent enantioselectivity α was then calculated as follows:

$$\alpha = k_2/k_1, \quad (4)$$

where k_2 is the retention factor for the more retained enantiomer (D-adenosine) and k_1 is the retention factor for the less retained enantiomer (L-adenosine). The efficiency of the column was characterized by estimating the number of theoretical plates N as follows:

$$N = 5.54(t_R/w_{50})^2, \quad (5)$$

where w_{50} is the peak width at half-height. The asymmetry factor A_s was determined by calculating the ratio of the second (or right part) of the peak to the first (or early part) of the peak at 10% of the peak height. The chromatographic resolution R_s was calculated using the following relation:

$$R_s = [1.18(t_{R2} - t_{R1})]/(W_{50(1)} + W_{50(2)}), \quad (6)$$

where the subscripts 1 and 2 refer to the first (nontarget) and last (target) enantiomers eluted.

Results and discussion

Evaluation of the properties of the covalently bonded anti-D-adenosine DNA CSP

In the first stage of this work, the properties of the covalently bonded DNA aptamer CSP were evaluated at ambient temperature (24 °C) and using as the mobile phase an aqueous buffer similar to that prepared for the SELEX procedure (20 mM phosphate buffer, 25 mM KCl, 1.5 mM MgCl_2 adjusted to pH 6.0). Frontal analysis chromatography was carried out in order to determine the active (available) binding sites (m_L) and the dissociation constant for the D-adenosine-immobilized aptamer association (K_d). Fig. 2 shows representative frontal analysis curves as well as the plot of $1/C(V_R - V_M)$ versus $1/C$ ($R=0.994$).

Using Eq. 2, we found m_L was to be 5.3 ± 0.5 nmol for a bed volume of 157 μL (column dimensions 20 cm \times 1-mm, inner diameter). Using an immobilization strategy based on the biotin–streptavidin bridge, Deng et al. [17] previously reported an anti-adenosine aptamer stationary phase characterized by a binding capacity of about 20 nmol for a bed volume of 100 μL . The lower binding capacity obtained here could be due to a coupling efficiency which would be

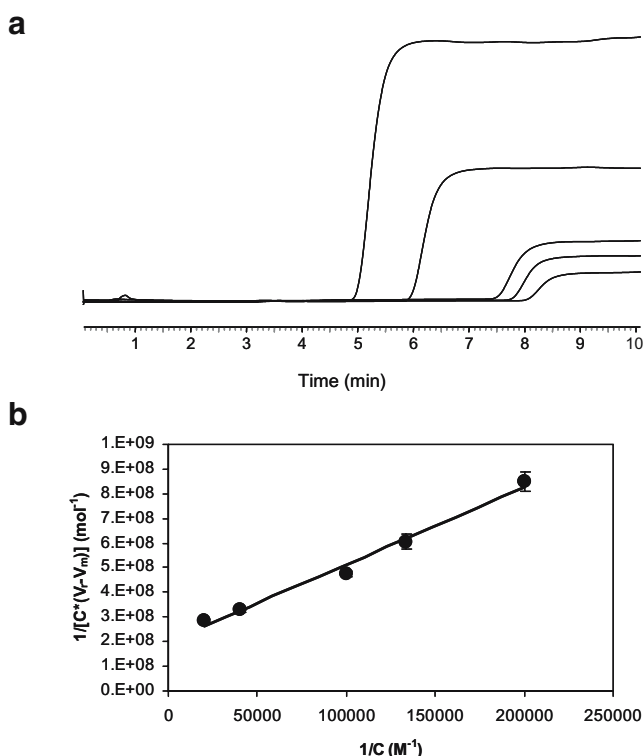


Fig. 2 (a) Frontal analysis chromatography elution profiles for D-adenosine on the adenosine-specific DNA aptamer chiral stationary phase (CSP) ($C=5\text{--}50\ \mu\text{M}$, from right to left). Column, $200\ \text{mm}\times 1.0\text{-mm}$ inner diameter; mobile phase, $20\ \text{mM}$ phosphate buffer, $25\ \text{mM}$ KCl, $1.5\ \text{mM}$ MgCl_2 adjusted to pH 6.0; flow rate, $50\ \mu\text{L}/\text{min}$; detection at $260\ \text{nm}$; $24\ ^\circ\text{C}$. (b) $1/[C(V_R - V_M)]$ versus $1/C$ for D-adenosine. In some cases, the error bar is obscured by the symbol

lower than that reported by Jarrett et al. [15, 16]. In addition, inefficient orientations of the CS onto the surface could be responsible for the low m_L value. Furthermore, it is well established that aptamers can adopt inactive conformations in relation to the medium composition or the temperature [7, 18]. The presence of a significant proportion of such conformers could also affect the binding capacity of the anti-adenosine aptamer column.

From the frontal analysis data, the K_d value for D-adenosine was estimated to be $17\pm 2.4\ \mu\text{M}$. This binding affinity was found to be on the same order of magnitude as those previously reported for two anti-adenosine aptamer stationary phases based on a biotin–streptavidin immobilization (14 ± 6 and $3\pm 1\ \mu\text{M}$) or for the anti-D-adenosine aptamer in solution ($6\pm 3\ \mu\text{M}$) [17, 19]. The discrepancy observed with two of these previously reported values could be due to the present immobilization process, which would affect the binding properties through some conformational changes of the aptamer binding sites. This could be also explained by the fact that (1) the aptamer sequence used in the present study was slightly different from that employed in the previous studies (see “Experimental”), (2) the operating conditions were not strictly identical and (3)

different methods were applied for the evaluation of the binding constant.

The enantioseparation properties of the DNA aptamer CSP were evaluated under the operating conditions selected for the frontal analysis. Fig. 3 shows a representative chromatogram obtained after injection of a racemic mixture of adenosine. The retention factors of D-adenosine and L-adenosine were found to be 2.53 ± 0.03 and 0.93 ± 0.02 , respectively. This was responsible for an apparent enantioselectivity α of 2.74 ± 0.08 . Under identical mobile phase and column temperature conditions, a similar apparent enantioselectivity has been observed using an anti-adenosine DNA aptamer column (bed volume of approximately $170\ \mu\text{L}$) based on a noncovalent biotin–streptavidin immobilization ($\alpha=3.44$) [3]. Concerning the band broadening and the peak shape, the number of theoretical plates per meter and the A_s values for the two enantiomers were estimated to be approximately 1,000–3,000 and approximately 1.30–1.50, respectively. As a comparison, the number of theoretical plates per meter observed for other target-specific bioaffinity CSPs such as antibody-based CSPs (using $5\ \mu\text{m}$ silica particles) varied from 1,500 to 6,000 in relation to the type of immobilization strategy [12]. Finally, the chromatographic resolution R_s was found to be 2.47 ± 0.32 in such operating conditions.

It can be concluded from both the frontal analysis and the zonal elution data that the covalent immobilization reported here constitutes a convenient approach as it allows the maintenance of suitable D-adenosine binding ability and chiral discrimination properties for an efficient enantioseparation.

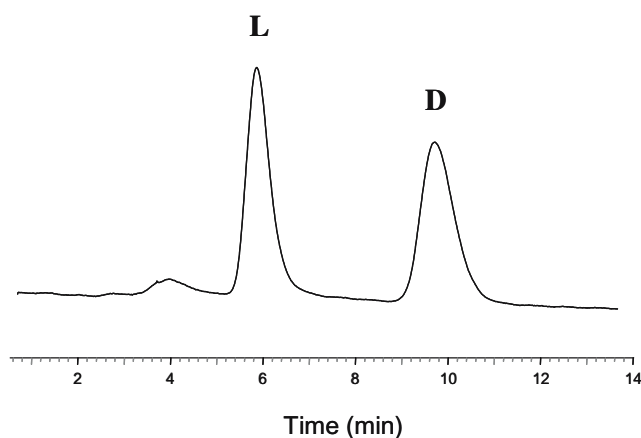


Fig. 3 Chromatogram obtained when a racemic mixture of adenosine was injected onto the adenosine-specific DNA aptamer CSP. Column, $200\ \text{mm}\times 1.0\text{-mm}$ inner diameter; mobile phase, $20\ \text{mM}$ phosphate buffer, $25\ \text{mM}$ KCl, $1.5\ \text{mM}$ MgCl_2 adjusted to pH 6.0; injection volume, $100\ \text{nL}$; flow rate, $50\ \mu\text{L}/\text{min}$; detection at $260\ \text{nm}$; $24\ ^\circ\text{C}$. L L-adenosine, D D-adenosine

Effects of operating chromatographic conditions on the enantioseparation

It is well established that monovalent and/or divalent cations are particularly important for inducing and stabilizing the active conformational structure of aptamers [20]. So, experiments were firstly performed to analyze the role of the mobile phase salt concentration on the binding and enantioseparation properties of the aptamer CSP. The MgCl_2 , KCl and sodium phosphate concentrations in the eluent were varied from 1.5–2 to 15–25 mM in relation to the type of salt. The solute retention factors and apparent enantioselectivity did not change significantly over the KCl and sodium phosphate concentration range. In contrast, an increase in the k and α values was obtained for the higher 15 mM Mg^{2+} concentration (compare Figs. 3, 6). Subsequently, a racemic mixture of adenosine was injected using a pure aqueous mobile phase (without any salt) after column equilibration with water for more than 20 column volumes. Surprisingly, it was found that the column performances were not altered under these conditions. The adenosine enantiomers were efficiently separated with chromatographic parameters similar to those obtained using the aqueous buffer as the eluent. It can be hypothesized from this result that some essential cations, involved in the stabilization of the active conformation of the aptamer, were not removed from the immobilized nucleic acid even after a column flushing with several water volumes. On the other hand, when the column was flushed with 20 column volumes of a sodium phosphate buffer (20 mM, pH 7.0) containing 5 mM EDTA, the enantioseparation was completely abolished under pure aqueous mobile phase conditions. The enantioselective properties of the DNA aptamer CSP were then totally restored by the passage of the buffered mobile phase containing 15 mM MgCl_2 (20 column volumes). All these data suggest that the presence of Mg^{2+} constitutes a major factor to promote and/or stabilize the active conformation of the aptamer. This is consistent with previous works which have shown that functional tertiary structures of nucleic acids are mainly dependent on the Mg^{2+} concentration [21, 22].

Additional experiments were performed using organic solvents of different polarities (methanol, ethanol, 2-propanol) as eluent modifiers. The proportion of alcohol modifiers in the aqueous buffer mobile phase was increased from 0 to 15–25% in relation to the type of organic solvent. Fig. 4 presents the variation of the adenosine chromatographic parameters as a function of the organic modifier fraction of the mobile-phase. These data revealed that, whatever the type of organic modifier, an increase in the amount of organic solvent was responsible for a decrease in the retention factor of the analyte and the resolution. On the other hand, the apparent enantioselectivity was slightly

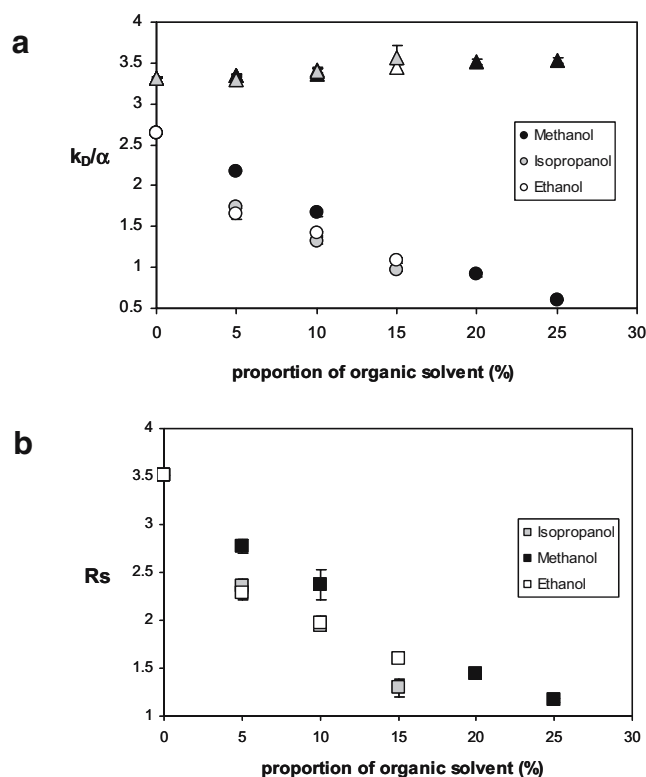


Fig. 4 (a) k_d (circles) and α (triangles) versus the proportion of organic solvent in the mobile phase. (b) R_s versus proportion of organic solvent in the mobile phase. In some cases, the error bar is obscured by the symbol. Column, 200 mm \times 1.0-mm inner diameter; mobile phase, 20 mM phosphate buffer, 25 mM KCl, 15 mM MgCl_2 adjusted to pH 6.0; injection volume, 100 nL; flow rate, 50 $\mu\text{L}/\text{min}$; detection at 260 nm; 24 $^\circ\text{C}$

enhanced. As an example, the α value increased from 3.31 ± 0.01 to 3.54 ± 0.03 when the proportion of methanol in mobile phase varied from 0 to 25%. Such results suggest that some hydrophobic effects were involved in the analyte-immobilized aptamer interaction but did not participate in the chiral discrimination mechanism. Solvent-dependent changes of the binding capacity or the active conformation of the immobilized DNA aptamer could also be responsible for the variation of the chromatographic parameters.

Further experiments were conducted over the 24–39 $^\circ\text{C}$ column temperature range using the aqueous buffer mobile phase. As shown in Fig. 5, the solute retention and the resolution were strongly reduced as the column temperature increased. A slight decrease in the apparent enantioselectivity was also observed (from 3.31 ± 0.01 to 3.00 ± 0.03). As previously reported [3, 6], this behavior could be explained by the involvement of enantiospecific interactions which were enthalpically driven and/or a temperature-dependent change of the conformation/binding capacity of the aptamer CSP.

From a practical point of view, it appears from these results that both the separation of the adenosine enantiomers and the analysis time can be easily modulated by

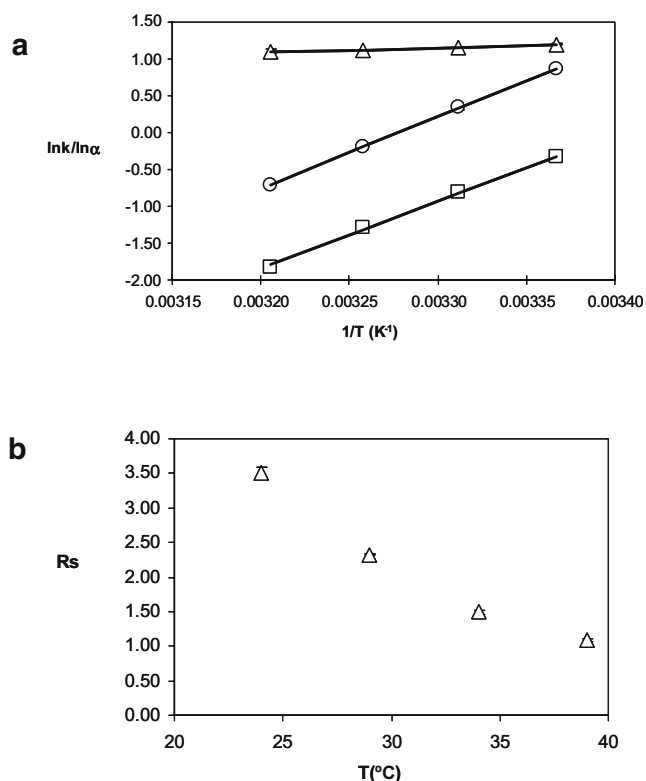


Fig. 5 (a) $\ln k/\ln \alpha$ (triangles) versus $1/T$ for D-adenosine (circles) and L-adenosine (squares). (b) R_s versus the column temperature. In some cases, the error bar is obscured by the symbol. Column, 200 mm \times 1.0-mm inner diameter; mobile phase, 20 mM phosphate buffer, 25 mM KCl, 15 mM MgCl₂ adjusted to pH 6.0; injection volume, 100 nL; flow rate, 50 μ L/min; detection at 260 nm

varying the composition of the mobile phase and the column temperature. The baseline resolution was achieved in a short analysis time (less than 10 min) by increasing (1) the column temperature to 34 °C or (2) the amount of organic modifiers (using, for example, 15% of methanol or ethanol in the buffered mobile phase).

Evaluation of the column stability

The stability of the aptamer CSP was evaluated during the course of this work under different chromatographic conditions, involving changes in the column temperature, eluent salt concentration and addition of organic modifiers to the mobile phase (see earlier). When not in use, the column was indifferently stored in the buffer containing sodium azide (0.05%) or in different buffer–organic solvent mixtures. The chromatographic performances of the column were compared in the initial conditions and after several experiments, using the buffered solution as the reference mobile phase. Fig. 6 presents two chromatograms obtained in the initial conditions and after the passage of more than 2,500 column volumes of the mobile phase. A slight increase in the solute retention factors was obtained

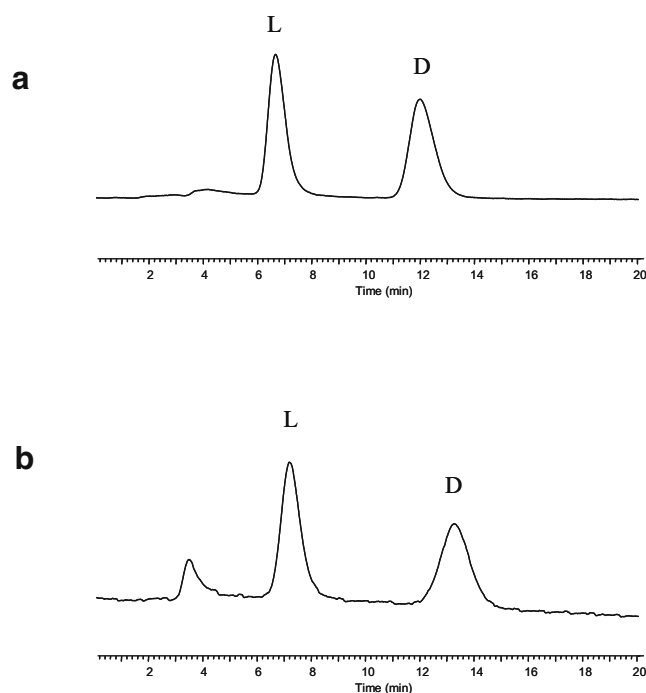


Fig. 6 Chromatograms obtained when a racemic mixture of adenosine was injected onto the adenosine-specific DNA aptamer CSP. (a) In initial conditions. (b) After the passage of more than 2,500 mobile phase column volumes. Column, 200 mm \times 1.0-mm inner diameter; mobile phase, 20 mM phosphate buffer, 25 mM KCl, 15 mM MgCl₂ adjusted to pH 6.0; injection volume, 100 nL; flow rate, 50 μ L/min; detection at 260 nm; 24 °C

(Fig. 6), without any change in the apparent enantioselectivity. This demonstrates the good stability of the covalently bonded aptamer CSP in a routine chromatographic use context.

Additional experiments were performed to evaluate the stability of the column under storage conditions traditionally employed for reversed-phase columns. The column was flushed with 20 column volumes of a pure aqueous mobile phase containing a high proportion of organic solvent (water–methanol, 50:50, v/v) and stored under these conditions for some days. The binding and enantio-separation properties of the aptamer CSP were found to be unaltered by such treatment.

In order to obtain further information about the stability of the stationary phase, the column was also tested in harsh operating conditions. The aptamer-based column was subjected to a very high temperature (60 °C) for 6 h, using a flow rate of 50 μ L/min. The column was then stored under a buffered mobile phase for 1 day at 4 °C. Although a loss in retention was observed (k_d was found to decrease by about 15%), the apparent enantioselectivity remained unchanged. The retention properties can be fully restored after about 1 week of storage under the buffered mobile phase.

Conclusion

In this paper, we have reported the preparation and the evaluation of a covalently bonded DNA aptamer CSP. The amide-bond-based covalent immobilization approach appears to be of interest as it allows one to obtain (1) valuable enantiodiscrimination properties and (2) good stability over the time, even under various conditions of use and storage. From a practical applicability point of view, these stability results suggest that this kind of covalent linkage constitutes a significant improvement relatively to the biotin–streptavidin link strategy previously used for the design of aptamer CSPs [2–6].

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