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Monolithic alkylsilane column: A promising separation medium for oligonucleotides by ion-pair reversed-phase liquid chromatography

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ABSTRACT

In this paper, a monolithic octadecylsilane column and particle-packed octadecylsilane columns were used to investigate the retention behaviors of oligonucleotides by ion-pair reversed-phase liquid chromatography (IP-RPLC). Results showed that, with same base composition, hairpin oligonucleotides always had weaker retention than corresponding random coil oligonucleotides on the monolithic column, but not on the particle-packed columns. In addition, the linear correlation between the retention factor *k* of oligonucleotides and the reciprocal of temperature (1/T), especially for hairpins, was relatively weaker on the particle-packed columns, as compared to the correlation on the monolithic column. The correlation between *k* and 1/T became weaker with decreasing particle size of the particle-packed columns. Moreover, results revealed that the overall retention order on the particle-packed column with small particles (3 μm) differed greatly from that on the monolithic column. In contrast, the retention order on the 10 μm particle-packed column was very close to that on the monolithic column. From the above, we inferred that oligonucleotides could keep their primary conformations unchanged when passing through the monolithic column, attributed to the special pore structures of the monolith. However, the conformations of oligonucleotides were suppressed or even destroyed when oligonucleotides passed through the particle-packed columns. This because the narrow and tortuous channels created by the stacked stationary phase particles could lead to more complex and unequable retention behaviors. Therefore, the monolithic column exhibited better retention regularity for oligonucleotides of secondary structure especially for hairpins than the particle-packed columns. It is noteworthy that the monolith-based IP-RPLC opens an intriguing prospect in accurately elucidating the retention behaviors of oligonucleotides.

1. Introduction

Being a carrier of genetic information, nucleic acids play a vital role in the continuation and evolution of life. The variety of composition, sequence and conformation result in the complexity of nucleic acids structure [1]. For example, the presence of inverted repeats in DNA or RNA sequences can lead to the formation of hairpins [1]. Apart from the biological functions [2–5], hairpin loops are attractive candidates for the design of antisense therapeutics [6–8]. As the market for nucleic acid based therapeutics and diagnostics grows, the demand increases for approaches for the isolation, characterization and purification of such materials [9]. However, the secondary structure and extraordinary conformational flexibility of nucleic acids pose key challenges to separation. Among various separation and detection methods, ion-pair reversed-phase liquid chromatography (IP-RPLC) has been proven to be a practical and popular tool for nucleic acid isolation and purification [10].

As the heart of chromatographic separation, column is the key to achieve effective separation of analytes in complex matrix. Nowadays, porous alkylsilane particle-packed columns are the most widely used commercial columns for the separation of nucleic acids by IP-RPLC [11–16], and the retention mechanisms of this kind of columns have been elucidated extensively [11,12,14,17–27]. It is noteworthy that, the later rising silica-based monolithic columns present high permeability and low flow resistance compared with particle-packed columns [28]. To realize rapid or efficient separation, silica-based monolithic columns have become the potential media for high performance liquid chromatography (HPLC) in

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the areas of environmental [29], pharmaceutical [30,31], clinical [32,33], industrial [34], and food analyses [35] in recent years. Studies have mainly focused on micromolecules but the use of silica-based monolithic columns for the separation of biopolymers is still very rare. For nucleic acids analysis, Moravcová et al. used two zwitterionic silica-based monolithic capillary columns coupled with tandem mass spectrometry to separate highly polar and hydrophilic nucleobases, nucleosides and nucleotides in hydrophilic interaction chromatography (HILIC) [36]. Kawamura et al. studied the retention behaviors of oligonucleotides on a monolithic column by capillary liquid chromatography (CLC) and on a particle-packed column by semi-μ-HPLC. They found that single-nucleotide polymorphisms (SNP) could be detected by CLC but not possible by semi-μ-HPLC. It was considered that the better resolution of CLC was primarily due to the sizing effect (the reduced column inner diameter and flow rate) and secondly due to the use of monolithic column [37]. However, in Kawamura's work, the influence of secondary structures of oligonucleotides on retention was ignored for CLC analysis, because a high column temperature of 60 °C was adopted [37]. Previous researches have indicated that secondary structures play an important role in the retention of oligonucleotides [38,39]. On the other hand, different structural forms are important for the participation in different biological functions of nucleic acids [40]. Therefore, understanding the retention behaviors of oligonucleotides on silica-based monolithic columns and particle-packed columns is very meaningful, in the sense of facilitating the reflection of structural information in solution of oligonucleotides especially for those with secondary structures such as hairpins.

In this present work, eight groups of oligonucleotides were selected as research objects, each group consisting of a hairpin and a random coil oligonucleotide. IP-RPLC retention behaviors of these oligonucleotides were investigated on one silica-based monolithic C18 column and four silica-based particle-packed C18 columns with different particle size. Retention characteristics of the studied oligonucleotides were first explored by determining the selectivity factor (α) between the hairpins and the random coils in all eight groups. Then, the relationship between oligonucleotides retention factor (k) and column temperature (T) was deduced systematically. The advantage of the monolithic column in the separation of oligonucleotides was concluded and the reason was explained by comparing the retention difference between monolithic and particle-packed columns.

2. Experimental section

2.1. Apparatus

Instrumentation for HPLC analysis was an LC-20AD consisting of a vacuum degasser, a binary high-pressure pump, an auto-sampler, a thermostatic column compartment, and a dual-wavelength UV detector (Shimadzu, Kyoto, Japan). The Labsolution software was used for the data acquisition and management. A FiveEasy Plus™ pH meter (Mettler-Toledo, Schwerzenbach, Switzerland) was used for the pH determination of buffer solutions.

2.2. Chemicals and materials

HPLC grade acetonitrile (CH_3CN) and triethylamine (TEA) were purchased from Honeywell (Ulsan, Korea) and TEDIA (Fairfield, USA), respectively. Acetic acid (≥ 99.5%, analytical reagent) was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Ultrapure water was used throughout the experiment.

Eighteen oligonucleotides were synthesized by Sangon Biotech (Shanghai, China), among which 16 hetero-oligonucleotides were used for retention behavior study (Table 1). Homo-oligonucleotides $(dA)_{12}$ and $(dT)_{10}$ were used as internal standards to normalize retention of test oligonucleotides via dual-point retention time correction [23]. All the oligonucleotides were dissolved in water with concentration of 10 μmol/l, and each of them were then mixed with same volume of $(dA)_{12}$ and $(dT)_{10}$ prior to HPLC injection.

Hetero-oligonucleotides were divided into eight groups. Each group had two oligonucleotides with the same base composition but different base sequences - one had a random coil structure and the other had a hairpin structure ($\Delta G < -3.5$ kcal/mol). The bases of hairpins in the first five groups were partially complementary, while in the remaining three groups, the bases were completely complementary. The inclination of oligonucleotide to form hairpin structure was calculated from http://www.idtdna.com/calc/analyzer.

2.3. Chromatographic conditions

Triethylammonium acetate (TEAA) was used as ion-pair reagent in mobile phase A (0.1 M TEAA containing 5% CH_3CN, pH 7.0) and mobile phase B (0.1 M TEAA, containing 25% CH_3CN, pH 7.0), and all mobile phases were filtered by 0.22 μm filter membrane before use. HPLC separation was accomplished on one silica-based monolithic column and four silica-based particle-packed columns at different flow rate, respectively (Table 2), with the same gradient elution run: 0–50 min, 10% B-60% B. Other chromatographic parameters were the same and given as following: column temperature, 25–45 °C; injection volume, 2 μl; UV detection wavelength, 260 nm.

2.4. Peak capacity and resolution calculation

Peak capacity (P_c) represents the maximum number of components that can be separated to unit resolution ($R_s = 1$) within a given time window under a given set of experimental conditions [41]. P_c is one of the most important metrics of separation quality in gradient elution [42,43]. For P_c calculation, there are two commonly used equations: $P_{c1}=1+t_G/W$ [42,44,45] and $P_{c2}=(t_{R,n}-t_{R,m})/W$ [44,46], where t_G is the gradient time; $t_{R,n}$ and $t_{R,m}$ are the retention times of the last and earliest eluting peaks, respectively; W is the average baseline peak width. P_{c1} calculation is based on the entire chromatogram, while P_{c2} calculation is based on the actual retention time window of eluted peaks.

The resolution (R_s) between two oligonucleotides were calculated according to $R_s = 2(t_{R2}-t_{R1})/(W_1+W_2)$, where t_{R1} and t_{R2} are the retention times of the former and the latter for two adjacent peaks; W_1 and W_2 are their peak widths at peak base.

3. Results and discussion

3.1. Retention behavior of oligonucleotides on monolithic column

To achieve fast separation, the maximum optimal mobile phase flow rate of 0.4 ml/min was adopted for the monolithic column. Narrow peak shape and excellent resolution were obtained for all the oligonucleotides at column temperature of 25 °C. The peak capacity (P_c) was calculated via P_{c1} and P_{c2}, respectively, to be 159 and 38. Representative chromatograms of oligonucleotides are shown in Fig. 1A. Table 3 shows that the hairpin and corresponding random coil oligonucleotides in each group were successfully separated with good resolution (R_s), and the biggest R_s appeared at group 8 (oligo 32). Notably, all hairpins had weaker retention than corresponding random coils. It is known that in IP-RPLC the retention of nucleic acids depends mainly on two interactions: electrostatic interaction and hydrophobic interaction [39,47], thus, both the number and the hydrophobicity of bases contribute to the retention of oligonucleotides. For hairpins, the complementary