**Temperature-independent Detection of Heteroduplex and Homoduplex Fragments Applying Poly(glycidylmethacrylate-co-divinylbenzene-based) Monoliths Modified to Strong Anion-exchanger**

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**Abstract**

Monoliths of poly(glycidylmethacrylate-co-divinylbenzene) were prepared in the confines of presilanized borosilicate glass columns (100 × 3 mm I.D.). These monoliths were surface modified into strong anion-exchangers with hydrochloric acid (10%) and triethylamine, successively. The strong anion-exchanger established good separation of 5-phospho-rylated oligodeoxythymidylic acids fragments \[ \text{d(pT)}_{12-18} \]. Moreover, heteroduplex and homoduplex fragments of a low-range mutation standard [of STS marker from the Y-chromosome (209 bp)] were separated at ambient and elevated temperatures using sodium phosphate buffer and a gradient former of sodium chloride, in anion-exchange high-performance liquid chromatography (AE-HPLC). This is a step forward for mutation detection as temperature-independent method, which is not the case in denatured ion-paired reversed-phase chromatography (D-IP-RP-HPLC), where mutation detection is temperature critical and might be bypassed if temperature changes slightly. Finally, reproducibility check from run-to-run and monolith-to-monolith showed a relative standard deviation (RSD) of less than 2%.

**Keywords:** Strong anion-exchanger (SAX); Monolith, Glycidylmethacrylate-co-divinylbenzene; Homoduplex and heteroduplex fragments.

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**1. Introduction**

High-performance liquid chromatography (HPLC) has been the most liable technique for separation of proteins and nucleic acids.\(^1\)-\(^4\) However, the high back-pressure and the long analysis time experienced have presented a setback for packed columns. Fast separation is feasible using monolithic stationary phases consisting of a single piece of a rigid macroporous polymer. Monoliths are distinguished for fast analysis due to the ample channels available, which allow high flow-rate at low pressure-drop, as well as high mass transfer due to enhancing convection and lessening diffusion.\(^5\)-\(^6\) Monoliths have been prepared from different materials such as inorganic silica by sol-gel\(^7\)-\(^9\) or organic polymer such as metal-catalyzed ring opening metathesis polymerization (ROMP),\(^10\) or free-radical polymerization based on styrene and acrylate chemistry.\(^11\)-\(^13\) The ease to make and derivatize, made these monoliths a good choice for present and future applications in bioseparation.

Ion-pair reversed-phase (IP-RP-HPLC) and anion-exchange (AE-HPLC) high-performance liquid chromatography are the most practical methods for nucleic acids separation.\(^14\)-\(^19\) Anion-exchange chromatography mode presents an efficient and environmentally benign method for proteins and nucleic acid analyses either for diagnostic or disease treatment purposes.\(^20\)-\(^21\) Moreover, AE-HPLC has been applied for mutation detection in dsDNA fragments that is of great importance in medicine, natural and social sciences. Such a separation of heteroduplex and homoduplex fragments of different mass ranges at different temperatures is accomplished.\(^16\),\(^22\) The separation of hetero- and homoduplex fragments using AE-HPLC monolithic column is advantageous to D-IP-RP-HPLC, where the latter is temperature critical. Minor discrepancy in the column temperature would lead to miss spotting of the mutation presence in the sample. This is not the case in AE-HPLC, where mutation is detected at a wide range of temperature. The dsDNA fragments (with
negative potential of the phosphate groups) are attracted to the positive potential of the anion-exchanger (ammonium groups).\textsuperscript{2,16}

Mutation presence is usually detected by the presence of four peaks in the chromatogram, two for homoduplex and two for the heteroduplex fragments, where the elution order depends on the method applied for its detection.\textsuperscript{12,14}

Few previous studies employed glycidylmethacrylate-co-divinylbenzene-based monoliths which were surface modified into weak and strong anion exchangers, taking advantage of the reactivity of the epoxy groups on the support surface.\textsuperscript{23–27} The benzene ring in DVB furnishes interaction with sodium hydroxide or phosphoric acid to the required pH. 5-phosphorylated oligodeoxynucleotide acids fragments [d(pT)\textsubscript{12-14}] sample was purchased from Sigma (Vienna, Austria). Low-range mutation standard [STS marker from the Y-chromosome (209 bp)] was purchased from Transgenomics (Berlin, Germany).

### 2. Instrumentation

HPLC-system of Transgenomic (Transgenomics, Berlin, Germany) was used in the analyses. The Transgenomic HPLC-system consisted of a pump, oven, autosampler, and UV-Vis detector. Nucleic acids were detected by UV-Vis detector at wavelengths of 260 nm. Wavemaker 4.0 (Transgenomics, Berlin, Germany) was used for data acquisition and processing. Nanopure infinity ultra-pure water and HPLC-grade acetonitrile were used in HPLC analyses. Borosilicate glass columns [Chromsep glass column (100 × 3 mm I.D.), catalogue number CP99912] were purchased from CP-Analytica (Vienna, Austria).

### 2.3. Silanization of Borosilicate Glass Columns

The borosilicate glass columns (100 × 3 mm I.D.) were silanized by sonication them in a mixture of ethanol/acetone (1/1, v/v), and then etched by soaking them in 2 mol/L KOH/ethylene solution overnight at a temperature of 60 °C. The following day, these columns were washed with plenty amount of water while sonication till reaching neutral effluents and dried under high vacuum for 6 h. A mixture, of 3-(trimethoxysilyl)propyl methacrylate (silanizing agent) and equal volume of 0.01% (w/v) 2,2-diphenyl-1-picylhydrazyl hydrate in dimethylformamide (DPPH/DMF) (as inhibitor to avoid temperature induced-polymerization of the silanizing agent), was filled into the glass columns, which were sealed using Eppendorf 2-ml vials and kept to react at temperature of 100 °C for 6 h. These columns were sonicated in acetone, water then ethanol for 15 min each, dried under high vacuum for 6 h and stored under argon for further use.

### 2.4. Monolith Preparation

Mixtures of specific combinations of glycidylmethacrylate, divinylbenzene, 1-decanol and THF (as macroporogen and microporogen, respectively) were prepared. 1% (w/v) of α,α’-azoisobutyronitrile (AIBN) was used to initiate polymerization. Each mixture was degassed and sonicated for 10 min, then poured inside the presilanized borosilicate glass column (100 × 3 mm I.D.). Then it was sealed on both sides by Eppendorf 2-ml vials. The column was left to polymerize for 24 h at 55 °C. Afterward, the seals were removed and the monolith was cut flat at both sides, fitting rings were placed and the monolith was placed in special stainless steel housing.
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The ion-capacity quantified by frontal analysis was 0.81 meq/g and by elemental analysis was 0.87 meq/g. These values are consistent showing a deviation of less than 10%.

3. 4. Separation of 5-phosphorylated Oligodeoxythymidylic Acids Fragments \([d(pT)_{12-18}]\)

The strong anion-exchange monolith synthesized was analyzed for separation of 5-phosphorylated oligodeoxythymidylic acids fragments \([d(pT)_{12-18}]\) to check its performance. The usual operating mobile phase pH’s applying strong anion-exchange columns is 7–9. As a starting point a phosphate buffer (0.02 M, pH 7, 20% ACN) and NaCl as a gradient former were used. Good separation of the seven fragments of oligodeoxythymidylic acids was accomplished in about 12 min (Fig. 2). Applying a gradient of sodium chloride as eluting salt (23–30)% in 4 min, then to 35% in 10 min at a flow-rate of 2 ml/min, the resolution of the seven fragments ranged from 1.2 to 1.46 (Table 1). To check the pH influence on separation of \(d(pT)_{12-18}\) phosphate buffers (0.02 M, 20% ACN, pHs 8.0 and 9.0) were used. When increasing the buffer pH from 7 to 8, applying the same conditions as mentioned above, the analysis time of \(d(pT)_{12-18}\) sample was decreased by 2 min and resolution was reduced by only ∼10% in average (Fig. 2, Table 1). When switching to buffer of pH 9, no significant influence was seen where the analysis time and the resolution were almost the same (Fig. 2, Table 1). This is in consistence with what was reported in a relatively recent study which stated that the higher the pH of the eluting buffer, the faster the elution is for \(d(pT)_{12-18}\) fragments. However, this contradicts what was reported by a previous study, which concluded a delayed elution of \(d(pT)_{12-18}\) fragments when increasing the pH of the eluting buffer.

This higher retention was rationalized on the base of an increasing deprotonation of the phosphate terminals of the oligonucleotides fragments at higher pH values, which
3.5. Separation of Heteroduplex and Homoduplex Fragments

3.5.1. Mutation & Mutation Samples

The low-range mutation sample (209bp) investigated in this study was a point mutation as AC, GT mismatch. The sample sequence is stated below, where the first underlined bold is the normal base and the second underlined bold is the variant base.

Low range mutation standard [STS marker from the Y-chromosome (209 bp)]
AGGCAACTGCTAGAATGAATGGGCACACA
GGACAAGTCCAGACCAGGAAATGTCGATTAACAT
GGGAGAAAGCGAGAAGGATTTCTAAAATTCAGG
GCTCCCTTTGGGCTCCCCTGTTTAAAAATGTAGG
TTTTATTATATATATTTATGTGTTAAACAAAGTCC
AGGTGAGATCTGTGGAGATAAAGGGGGCTGT
ATTTTCATT

The entirely complementary base sequence of both strands are called a homoduplex; while a duplex that contains at least one base pair, which is not complementary (mismatch), is called a heteroduplex.16,30 The most common mismatches in heteroduplex are AC and GT,31 which is the case in the STS marker from the Y-chromosome.

3.5.2. Analysis of Mutation Applying Anion-exchanger Monolith

In this study, a mutation sample was analyzed at room and elevated temperatures, on anion-exchanger support to compare the chemically and thermally induced denaturation of the double-stranded dsDNA. The chemical-induced denaturation is due to interaction between the dsDNA and the buffer solution. Low-range mutation sample was analyzed at ambient temperature applying phosphate buffer (0.02 M, pH 8), and sodium chloride (1.0 M) as gradient former. The four peaks of homoduplex and heteroduplex fragments were detected (Fig. 3a). The peaks of homoduplex were clearly resolved from those of heteroduplex.

To check the temperature influence on separation of heteroduplexes from homoduplexes, temperature was elevated from 25 to 40, 55, and 70 °C (Fig. 3b–d) maintaining identical chromatographic conditions. Increasing the column temperature to 40 and 55 °C lead to better separation of AT from GC (of the homoduplex fragments), and AC from GT (of the heteroduplex fragments), (Fig. 3b,c). At column temperature of 70 °C, it was noticed that the homoduplex AT and the heteroduplex GT started to coelute (Fig. 3d). Nevertheless, the four peaks were always seen at the four temperatures studied, which is tentative for mutation presence. Furthermore, applying 5% acetonitrile as organic modifier (Fig. 3e), maintaining similar chromatographic conditions applied in Fig. 3b, improved the resolution of the four peaks of AT, GC, AC and GT. Finally, decreasing the flow-rate from 1.0 to 0.5 mL/min at temperature of 25 °C demonstrated good separation of the four fragments AT, GC, AC, and GT (Fig. 3f) but doubled the analysis time. To sum up, the four peaks were seen clearly at all conditions applied, which assure no bypass of the mutation detection, applying this proposed method. The change in analysis temperature caused minor improvement in separating the homo- and heteroduplex peaks, which is a result of two effects: First, it enhances denaturation of the dsDNA (of the heteroduplex fragments), and second, the higher temperatures cause the dsDNA ladder to flatten (uncurl) and consequently lead to higher interaction with the support, and again better resolution.1–6,25–28

Table 1. Comparison of retention time (tR), peak half width (W0.5) and resolution (Rs) of d(pT)12–18 fragments on strong anion-exchange DVB-GMD-based monolith (100 × 3 mm I.D.). Mobile phase, A: 0.02 mol/L sodium phosphate (pHS 7, 8, & 9, 20% acetonitrile); B: mobile phase A + 1.0 mol/L sodium chloride; gradient, 23–30% B in 4 min, then to 35% in 10 min; flow-rate, 2 mL/min; temperature, 25 °C; sample, 5.0 μg d(pT)12–18.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>pH 7</th>
<th>pH 8</th>
<th>pH 9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tR</td>
<td>W0.5</td>
<td>Rs</td>
</tr>
<tr>
<td>d(pT)12</td>
<td>1.91</td>
<td>0.50</td>
<td>1.46</td>
</tr>
<tr>
<td>d(pT)13</td>
<td>3.16</td>
<td>0.51</td>
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<td>d(pT)14</td>
<td>4.62</td>
<td>0.77</td>
<td>1.20</td>
</tr>
<tr>
<td>d(pT)15</td>
<td>6.13</td>
<td>0.73</td>
<td>1.20</td>
</tr>
<tr>
<td>d(pT)16</td>
<td>7.64</td>
<td>0.77</td>
<td>1.21</td>
</tr>
<tr>
<td>d(pT)17</td>
<td>9.18</td>
<td>0.74</td>
<td>1.15</td>
</tr>
<tr>
<td>d(pT)18</td>
<td>10.68</td>
<td>0.80</td>
<td>1.35</td>
</tr>
</tbody>
</table>

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3.5.3 Elution Order of the Mutation Sample Fragment

Heteroduplex fragments tend to thermally denature more extensively than their corresponding perfectly matched homoduplex fragments in D-IP-RP-HPLC. Consequently, they are retained less and elute in front of the homoduplexes.

Moreover the fragments rich in AT-base pairs are eluted prior to GC-base pairs. So the order of elution is AC, GT, AT, GC in D-IP-RP-HPLC.\textsuperscript{1,30} Applying AE-HPLC, the dsDNA molecules begin to partially denature chemically by the electrolytic buffer present. The elution order of the heteroduplex and homoduplex fragments in the mutation sample is reversed in AE-HPLC as the latter precedes for-
monomer fragments in elution,\textsuperscript{16} where the elution mode is different in AE- from that of RP-IP-HPLC, esp. when NaCl is applied as a gradient former. Both size and type dependent of the DNA sequence influence the elution order,\textsuperscript{1–2,32} but since the mutation fragments are of identical size, then their elution order would be influenced by the sequence type only, so GC will elute before AT \textsuperscript{1–2,22,33} and the elution order would be as GC, AT, GT, then AC. (Scheme 2).

3. 6. Monolith Reproducibility, Storage and Regeneration

The run-to-run monolith reproducibility was studied by running 15 sets of runs as four blanks followed by one sample run at the same conditions, then the column was flushed overnight by water and was conditioned the following day by mobile phase for 1 h, and another set of analyses was done as the day before. The reproducibility of run-to-run in retention time was of relative standard deviation (RSD) of 2, 1, 2, and 1\% for the AC, GT, AT, and GC, respectively). Monolith-to-monolith reproducibility was done by comparing the retention times of d(pT)\textsubscript{12–18} sample for two monoliths made separately and derivatized applying the same procedure. The RSD in retention time was found to be 3, 4, 4, 2, 2, 2, 2\% for d(pT)\textsubscript{12–18} fragments, respectively.

The monolith was stored in methanol for two months, then for recheck it was washed with 200 ml of water followed by 200 ml of a mixture of 0.3 M sodium acetate and 0.2 M sodium dihydrogen phosphate to activate it. Then the monolith was flushed with mobile phase overnight. After storage the monolith did not show any decay in its efficiency, and the change in retention times of d(pT)\textsubscript{12–18} fragments was less than 0.05 min.

4. Conclusion

Strong anion-exchange monoliths were prepared applying a new method of surface modification of glycidylmethacrylate. The monolith prepared established good separation of 5-phosphorylated oligodeoxythymidylic acids fragments [d(pT)\textsubscript{12–18}], and tentative detection of mutation at room applying phosphate buffer and NaCl as former gradient. The four fragments of the homoduplex and heteroduplex were separated at various temperatures establishing a reliable method to detect mutation with no potential failure to spot. This makes AE-HPLC a complementary approach to D-IP-RP-HPLC method in mutation detection. Moreover, these monoliths showed high stability and reproducibility.

5. References


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Povzetek

Znotraj predhodno silaniziranih borosilikatnih steklenih kolon (100 × 3 mm I.D.) smo pripravili monolite poli(glicidil-metakrilat-co-divinilbenzen)-a. Te monolite smo na površini modificirali v močne anionske izmenjevalce najprej s klorovodikovo kislinjo (10%) in nato s trietilaminom. Z močnim anionskim izmenjevalcem smo dosegli dobro ločbo fragmentov 5-fosforiliranih oligodeoksitimidilnih kislin [d(pT)12–18]. Poleg tega smo lahko pri sobni in pri povišani temperaturi ločili heterodupleks in homodupleks fragmente standarda za nizko mutacijo [STS marker pri Y-kromosomu (209 bp)], pri čemer smo za anionsko izmenjavo visokoeljivostno tekočinsko kromatografijo (AE-HPLC) uporabili pufer natrijev fosfat in natrijev klorid za nastanek gradienta. To je korak naprej proti detekciji mutacij s temperaturno neodvisno metodo, kar sicer ni slučaj pri denaturirani ionsko-parni kromatografiji na reverzni fazi (D-IP-RP-HPLC), pri kateri je detekcija mutacij kritično odvisna od temperature in jih je možno spregledati, če se temperatura nekoliko spremeni. Končni preizkus obnovljivosti od analize do analize (“run-to-run”) in od monolita do monolita je pokazal relativni standardni odklon (RSD) manj kot 2%.