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Studying the mechanism of RNA separations using RNA chromatography and its application in the analysis of ribosomal RNA and RNA:RNA interactions

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ABSTRACT

DNA/RNA chromatography presents a versatile platform for the analysis of nucleic acids. Although the mechanism of separation of double stranded (ds) DNA fragments is largely understood, the mechanism by which RNA is separated appears more complicated. To further understand the separation mechanisms of RNA using ion pair reverse phase liquid chromatography, we have analysed a number of dsRNA and single stranded (ss) RNA fragments. The high-resolution separation of dsRNA was observed, in a similar manner to dsDNA under non-denaturing conditions. Moreover, the high-resolution separation of ssRNA was observed at high temperatures (75 °C) in contrast to ssDNA. It is proposed that the presence of duplex regions/secondary structures within the RNA remain at such temperatures, resulting in high-resolution RNA separations. The retention time of the nucleic acids reflects the relative hydrophobicity, through contributions of the nucleic sequence and the degree of secondary structure present. In addition, the analysis of RNA using such approaches was extended to enable the discrimination of bacterial 16S rRNA fragments and as an aid to conformational analysis of RNA. RNA:RNA interactions of the human telomerase RNA component (hTR) were analysed in conjunction with the incorporation of Mg^{2+} during chromatography. This novel chromatographic procedure permits analysis of the temperature dependent formation of dimeric RNA species.

1. Introduction

The role of RNA recognition is assuming increasing significance in biological systems. In addition to its role in biological catalysis, recently exemplified by the structural studies on the ribosome, small RNA molecules are emerging as key regulators of gene expression in both prokaryotes [1], higher organisms (reviewed in Ref. [2]) and are also associated with mediating antiviral response in prokaryotes [3,4]. The synthesis, purification and analysis of RNA transcripts are key steps in the investigation of such biological events. Currently polyacrylamide gel electrophoresis (PAGE) is the principle method for defining the structural homogeneity of RNA transcripts and for monitoring the purification of chemically synthesised RNA for *in vitro* studies [4–6]. This procedure is time consuming and suffers from labour intensity, poor product yields and is unsuitable for high throughput approaches. More recently, capillary electrophoresis-laser induced fluorescence (CE-LIF) has been used to analyse RNA conformation, demonstrating advantages in sensitivity and reduced analysis times [7]. However, fluorescent labeling of the nucleic acid is required prior to analysis.

RNA/DNA chromatography is now established as a versatile technique for the analysis of nucleic acids [8]. This form of HPLC analysis is largely based upon the unique separation properties of a non-porous polystyrene-divinylbenzene polymer bead that has been functionalised with C18 alkyl groups. An alkylammonium salt is added to the eluent and forms neutral ion pairs when a DNA sample is introduced into the HPLC instrument. A gradient of acetonitrile solvent separates the nucleic acid fragments with the smaller fragments eluting from the column first. It has previously been used in the sequence independent sizing of duplex DNA (up to 2000 base pairs (bp)) under non-denaturing conditions [9] and using denaturing conditions, the analysis of oligonucleotides [10], the enrichment, separation and analysis of RNA [11–14]. RNA/DNA chromatography also provides a versatile platform for the rapid analysis of a wide range of nucleic acid modification reactions [15]. Further developments have also demonstrated the ability of RNA/DNA chromatography to indirectly study RNA conformation and DNA–protein interactions by analysing the products of DNA and RNA footprinting reactions [16–18].

Although the mechanism of separation of double stranded (ds) DNA fragments is largely understood, the mechanism by which RNA is separated appears more complicated. To further understand the separation mechanisms of RNA using ion pair reverse phase liquid chromatography, we have analysed a number of dsRNA and

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single stranded (ss) RNA fragments. In addition, the analysis of RNA using such approaches was extended to enable the discrimination of bacterial 16S rRNA and the direct analysis of RNA:RNA interactions exemplified by the RNA component of human telomerase (hTR).

2. Materials and methods

2.1. HPLC analysis

All samples were analysed by IP-RP-HPLC on an Agilent 1100 HPLC (Agilent, Palo Alto, CA, USA) using a DNAsep column 50 mm × 4.6 mm I. D. (Transgenomic, San Jose, CA, USA). The stationary phase of the column consists of a 2-μm I. D. non-porous, alkylated poly(styrene-divinylbenzene) matrix. Chromatograms, analysed using UV detection, were recorded at a wavelength of 260 nm.

2.2. RNA/DNA chromatography

The chromatographic analysis was performed using the following conditions: buffer A 0.1 M triethylammonium acetate (TEAA) (Fluka, UK), pH 7.0; buffer B 0.1 M TEAA, pH 7.0 containing 25% acetonitrile. The HaeIII digest of pUC18 (250 ng/μl 5 μl injected) (Bioline, London, UK) and the dsRNA ladder (300 ng/μl 5 μl injected) (New England Biolabs (NEB), Hitchin, Herts, UK) was analysed at 50 °C using the following linear gradient (1): starting at 10% buffer B the gradient was extended to 20% buffer B in 2.5 min, followed by a linear extension to 40% buffer B over 2.5 min followed by a linear extension to 70% buffer B over 13 min at a flow rate of 1.0 ml/min. At 75 °C the dsRNA ladder and HaeIII digest of pUC18 was analysed using gradient (1).

The total RNA and ssRNA ladder was analysed using gradient (2) starting at 20% buffer B the linear gradient was extended to 22% buffer B in 2 min, followed by a linear extension to 52% buffer B over 15 min, followed by a linear extension to 65% buffer B over 2.5 min at a flow rate of 1.0 ml/min

The bacterial rRNA was analysed at 50 °C using gradient (3) starting at 35% buffer B the linear gradient was extended to 50% buffer B in 3 min, followed by an extension to 65% buffer B over 15 min at a flow rate of 1.0 ml/min. Analysis of the hTR RNA was performed over a range of temperatures using the following gradient (4): starting at 30% buffer B the linear gradient was extended to 60% buffer B over 10 min at flow rate of 1.0 ml/min.

2.3. Polyacrylamide gel electrophoresis (PAGE)

5–10% non-denaturing polyacrylamide gels were prepared using acrylamide:bisacrylamide 29:1 (Bio-Rad, Hemel Hempstead Herts, UK) buffered with 45 mM Tris–borate (pH 8.0), 1 mM EDTA and run for 1 h, 100 V at 25 °C. PAGE gels containing magnesium were buffered with 45 mM Tris–borate (pH 8.0), 1 mM $MgCl_2$ and run for 1 h, 100 V at 25 °C. The gels were visualised after staining with fluorescent dyes SYBR green for dsDNA (Sigma, UK) using a Transilluminator.

2.4. In vitro transcription of hTR

The plasmid pGRN164 (a kind gift from Geron, Menlo Park, CA, USA) was linearised using FspI (NEB) prior to *in vitro* transcription. 2 μg of template DNA was incubated overnight at 37 °C and transcribed using a T7 Megascript *in vitro* transcription kit (Applied Biosystems, Warrington, Cheshire, UK) following the manufacturers instructions. The RNA was then precipitated in ethanol and ton, Cheshire, UK). Analysis was then performed using either 4.5% denaturing (8 M urea) PAGE or IP-RP-HPLC.

2.5. Folding of RNA transcripts

Following transcription, RNA samples were purified using denaturing gel polyacrylamide gel electrophoresis in 7 M urea. RNA was eluted from the gel by the addition of 2 volumes of 0.5 M ammonium acetate (Sigma, UK), 0.1% SDS (Sigma, UK) and 2 mM EDTA (Sigma, UK). The RNA was subsequently precipitated in ethanol and re-suspended in 10 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$ and 40 mM NaCl. To ensure folding of the RNA, samples were heated to 75 °C for 1 min and cooled slowly to 25 °C (2 °C/min).

2.6. Bacterial growth and RNA extraction

Escherichia coli K12 (NEB, Hitchin, Herts, UK), *Salmonella enterica* (ATCC 700720) and *Pseudomonas putida* (ATCC 49128) were grown to mid-log phase (A_{600} = 0.4–0.6) in Luria–Bertani (LB) media and nutrient broth (DIFCO) respectively, with rapid shaking and incubation at 37 °C. Cell pellets were obtained using centrifugation (5 min 10,000 × *g*) and washed with 1 ml phosphate buffered saline (1× PBS). The total RNA was extracted using Ribopure Bacteria Kit (Applied Biosystems, Warrington, Cheshire, UK) following the manufacturers instructions. RNA was re-suspended in RNase free water prior to HPLC analysis.

3. Results and discussion

3.1. Elucidating the mechanism of RNA separations using RNA chromatography

The rapid, high-resolution separation of ssRNA using denaturing RNA/DNA chromatography has been previously reported [11–13], offering significant advantages compared to gel electrophoretic analysis of RNA. In this study we have analysed the separation of a range of dsRNA transcripts (21–500 bp) using RNA chromatography at 50 °C (see Fig. 1A). The results demonstrate the rapid, high-resolution separation of dsRNA molecules in a similar manner observed to the separation of dsDNA using IP-RP-HPLC. However, a number of significant differences between the separation of dsDNA and dsRNA are observed. A direct comparison of the IP-RP-HPLC analysis of the dsRNA marker (21–500 bp) and the dsDNA marker, pUC18 HaeIII digest (80–587 bp) analysed under the same chromatographic conditions (see Section 2) is shown in Fig. 1A and B respectively. The dsRNA fragments can be seen to elute earlier than the corresponding dsDNA species of the same size. We have previously observed that dsDNA fragments containing uracil instead of thymine elute at slightly earlier retention times, demonstrating that sequence specific effects can alter the retention time of dsDNA [18]. However, the large differences in retention time observed between the dsRNA and dsDNA fragments cannot completely be accounted for by the small differences in hydrophobicity of thymine and uracil. dsDNA is known to adopt B-DNA conformation, whereas dsRNA adopts an alternative A-DNA conformation. These differences in structure may also be reflected in the difference in retention times observed between dsRNA and dsDNA fragments under the chromatographic conditions. The A-DNA conformation is essentially a shorter squatter version of B-DNA and this difference in structure may reflect a decrease in overall hydrophobicity compared to dsDNA. These results are consistent with the previous analysis of non-canonical B-DNA structures using DNA chromatography. Holliday junctions which adopt non-uniform tertiary structures were previously analysed using DNA chromatography and showed a decrease in hydrophobicity and therefore

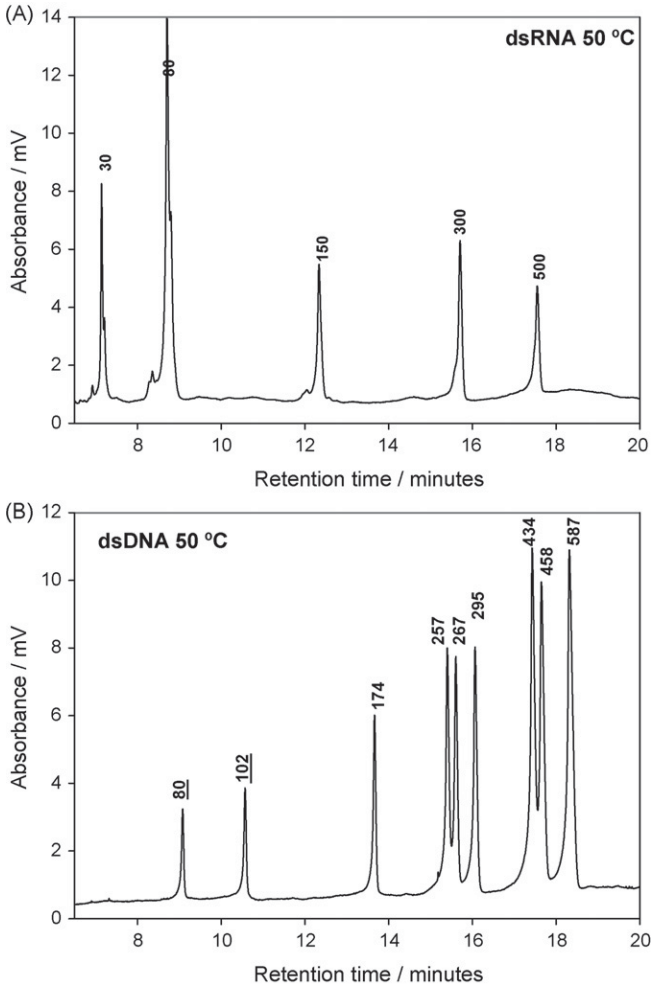

Fig. 1. IP-RP-HPLC analysis of dsDNA and dsRNA at 50 °C. (A) dsRNA marker. (B) pUC18 HaeIII digest. The size of the nucleic acid fragments in base pairs are highlighted. The samples were analysed using gradient (1). Buffer A: 0.1 M TEAA, pH 7.0; buffer B: 0.1 M TEAA, pH 7.0, 25% acetonitrile. 10–20% B in 2.5 min, 20–40% B in 2.5 min, 40–70% B in 13 min at a flow rate of 1.0 ml/min at 50 °C.

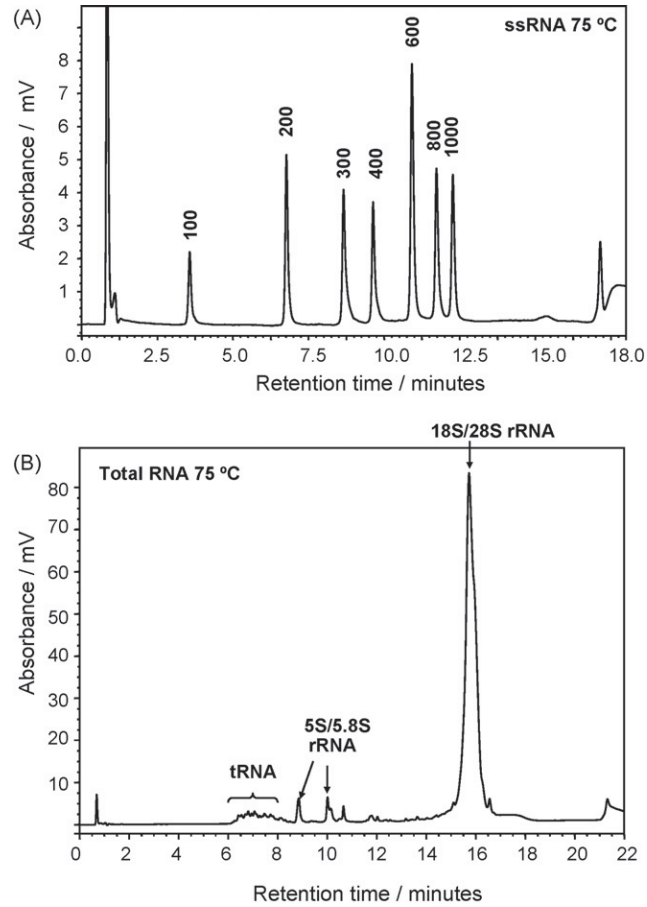

Fig. 2. IP-RP-HPLC analysis of RNA. Chromatograms show the separation of (A) ssRNA marker and (B) total RNA extracted from mammalian cells. The size of the nucleic acid fragments of the RNA marker in nucleotides and the total RNA species are highlighted. The samples were analysed using gradient (2). Buffer A: 0.1 M TEAA, pH 7.0; buffer B: 0.1 M TEAA, pH 7.0, 25% acetonitrile. 20–22% B in 2 min, 22–52% B over 15 min, 52–65% B over 2.5 min at a flow rate of 1.0 ml/min at 75 °C.

weight [18]. In addition, the co-axial stacking of helicies in such structures in the presence of magnesium ions also caused a change in retention time [18]. Furthermore, RNA is known to adopt more stable secondary/tertiary conformations in comparison to ssDNA which maybe present under IP-RP-HPLC conditions and contribute to the decrease in hydrophobicity compared to B-DNA duplex fragments.

3.2. Analysis of ssRNA under different temperatures

The high-resolution separation of ssRNA at high temperature is demonstrated in the analysis of a ssRNA size marker (100–1000 nt) shown in Fig. 2A. The results demonstrate what appears to be the size dependent separation of RNA at high temperature consistent with previous observations [11–13]. However, a number of aberrant retention times were observed in the analysis of RNA, which has also been observed in the analysis of ssDNA molecules (<100 nt) under fully denaturing conditions [9]. Such aberrant retention times of RNA can be clearly observed in the analysis of total RNA extracted from mammalian cells (see Fig. 2B). The chromatogram shows the co-elution of the 18S rRNA and 28S rRNA (1869 and 5035 nt respectively) at 75 °C. Collection of the corresponding peak and analysis using denaturing PAGE confirmed the presence of both the 18S and completely size dependent) in the analysis of the high molecular weight ssRNA transcripts under denaturing conditions were unexpected. It was anticipated that ssRNA would be similar to ssDNA in behaviour, where a decrease in resolution is observed (compared to dsDNA) as the molecular weight of the fragments increases. The loss of resolution in the analysis of ssDNA fragments is clearly observed in the analysis of dsDNA pUC18 HaeIII digest at 75 °C (see Fig. 3A). The chromatogram shows the loss of resolution of the individual DNA fragments. In contrast, the analysis of the dsRNA transcripts at 75 °C is shown in Fig. 3B. The chromatograms show clear differences in the resolution of ssRNA fragments compared to ssDNA fragments at high temperatures using RNA/DNA chromatography. The results demonstrate the loss of resolution of ssDNA at high temperatures compared to dsDNA at 50 °C (see Fig. 1B vs Fig. 3A). However, the analysis of the dsRNA at 75 °C reveals that each RNA duplex is subsequently separated into the ssRNA fragments which are resolved under the chromatographic conditions, enabling resolution of each separate species within each duplex. For clarity the resolution of the 80 bp RNA transcript is not shown on the figure, as the mass of this fragment in the marker is twice that of the other fragments. These results demonstrate that high-resolution separation of the ssRNA is achieved at high temperatures. However, such separations are not completely size dependent, as each of the two ssRNA fragments generated of the same size from each dsRNA duplex are

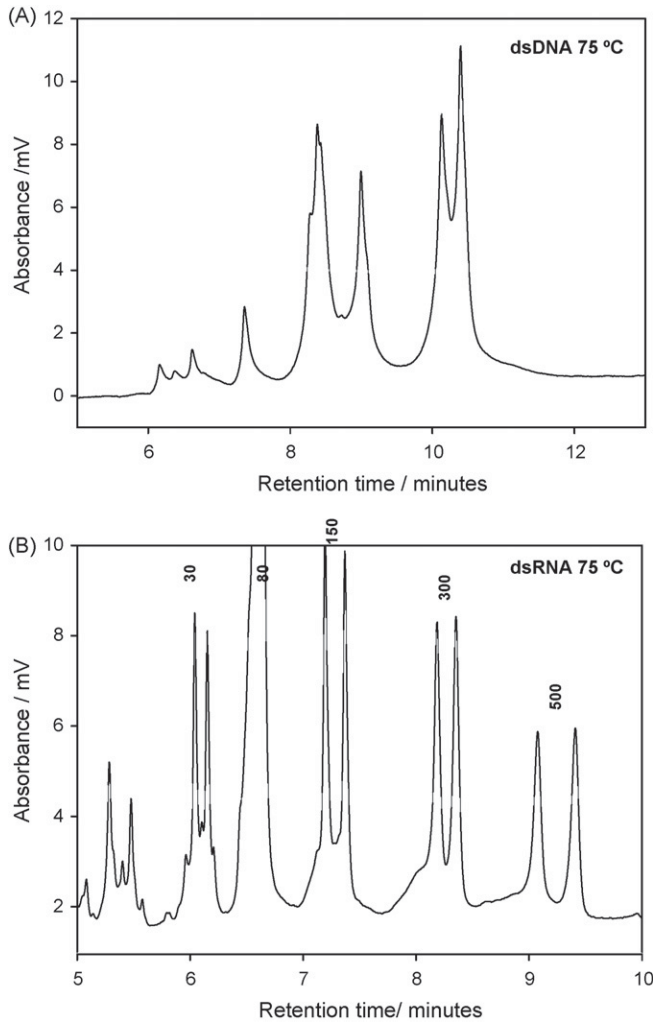

Fig. 3. IP-RP-HPLC analysis of dsDNA and dsRNA at 75 °C. (A) pUC18 HaeIII digest (B) dsRNA marker. The size of the nucleic acid fragments in nucleotides are highlighted. The samples were analysed using gradient (1). Buffer A: 0.1 M TEAA, pH 7.0; buffer B: 0.1 M TEAA, pH 7.0, 25% acetonitrile. 10–20% B in 2.5 min, 20–40% in 2.5 min, 40–70% B in 13 min at a flow rate of 1.0 ml/min at 75 °C.

3.3. Analysis of bacterial rRNA

The rapid and accurate detection of micro-organisms is an important tool in a wide range of applications including environmental monitoring, molecular diagnostics and food monitoring. rRNA in bacterial cells is often used as a target for bacterial identifications and discriminations due to the relative abundance and availability of 16S rRNA sequences [19–21]. Furthermore, due to sequence variability, it is often possible to use rRNA probes to classify bacterial species (reviewed in Ref. [22]). 16S rRNA sequences for *E. coli* K12, *S. enterica* and *P. putida* were obtained from the NCBI Entrez Genome Project. Each of the bacterial sequences has 7 operons including a number of sequence differences between the individual operons. The 16S rRNA from *E. coli* (1534 nt) was aligned with *P. putida* (1518 nt) revealing 85% sequence identity between *E. coli* and *P. putida*. Alignment of *E. coli* with *S. enterica* (1542–1546 nt) revealed >97% similarity. Total RNA was extracted from *E. coli* and *P. putida* (see Section 2) and analysed using RNA chromatography. The resulting chromatograms are shown in Fig. 4. Each bacterial total RNA was run in quadruplicate and the retention times of the 16S rRNA highlighted, including the standard deviations. The result demonstrates the ability of RNA chromatography

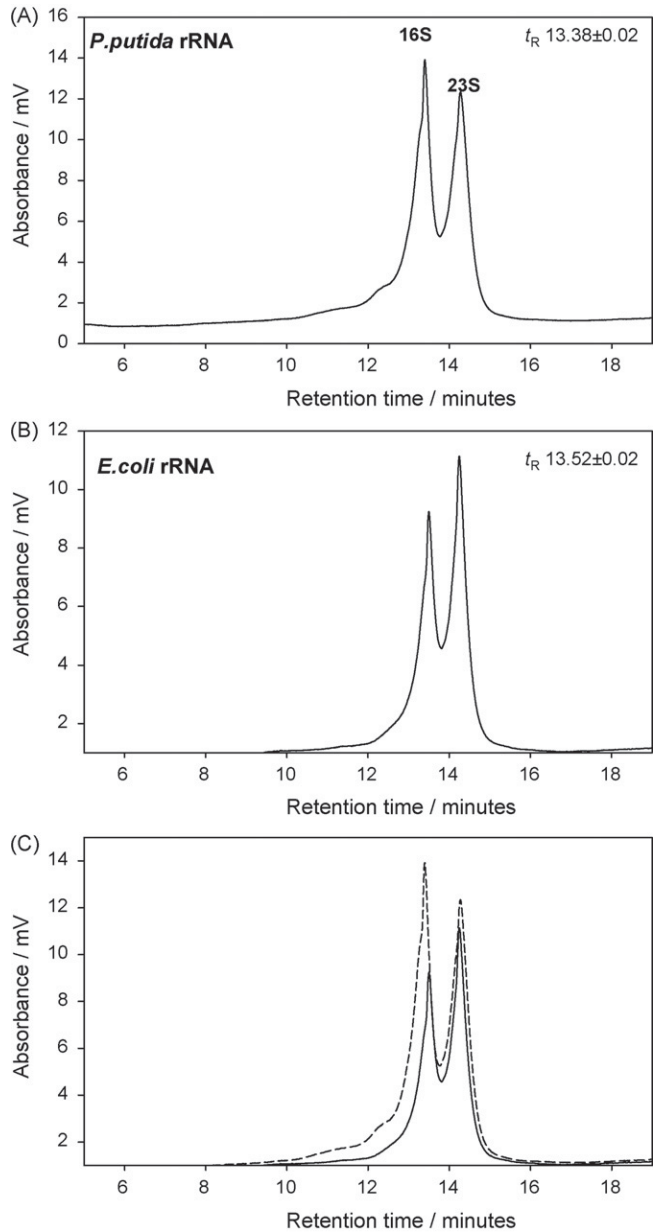

Fig. 4. IP-RP-HPLC analysis of bacterial ribosomal RNA. Chromatograms show the analysis of RNA extracted from (A) *P. putida* and (B) *E. coli*. The retention times of the 16S rRNA are highlighted from quadruplicate injections including the standard deviation. (C) Chromatogram of an overlay of the *E. coli* and *P. putida* rRNA. The bacterial rRNA was analysed at 50 °C using gradient (3). Buffer A: 0.1 M TEAA, pH 7.0; buffer B: 0.1 M TEAA, pH 7.0, 25% acetonitrile. 35–50% B in 3 min, 50–65% B in 15 min at a flow rate of 1.0 ml/min.

bacterial 16S rRNA (1534 nt vs 1518 nt), following the rapid extraction and analysis of the 16S rRNA, by virtue of differences in the retention time. No downstream manipulation, design or synthesis of probes was necessary. Such studies could also be extended in the analysis of complex microbial communities. The chromatography was unable to distinguish *E. coli* and *P. putida* 16S rRNA which share >97% similarity (data not shown).

3.4. Analysis of human telomerase RNA under non-denaturing conditions

Human telomerase is a ribonucleoprotein complex comprising two essential components, a catalytic protein subunit (hTERT) and

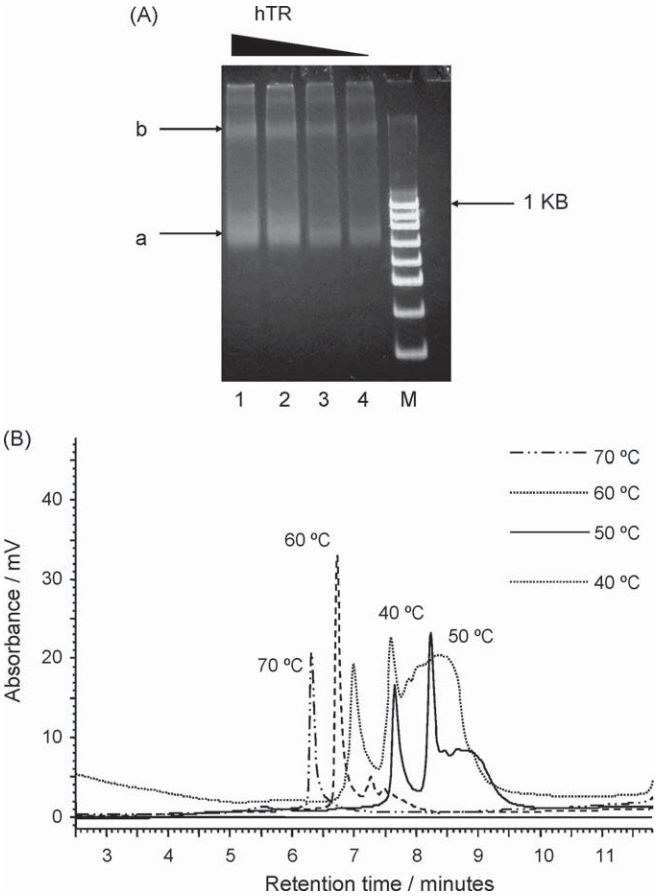

Fig. 5. Electrophoretic and chromatographic analysis of hTR. (A) Non-denaturing PAGE analysis of hTR. Electrophoretogram showing the presence of two different hTR species when analysed under non-denaturing conditions at 25 °C. Lanes 1–4 contain *in vitro* transcribed hTR, the two main RNA species are indicated (a and b). Lane M contains a 100-bp duplex DNA ladder. (B) IP-RP-HPLC analysis of hTR in the presence of Mg^{2+} ions. Chromatogram showing the temperature dependent analysis of hTR in the presence of 1 mM Mg^{2+} ions. The samples were analysed using gradient (4). Buffer A: 0.1 M TEAA, pH 7.0; buffer B: 0.1 M TEAA, pH 7.0, 25% acetonitrile. 30–60% B in 10 min at flow rate of 1.0 ml/min.

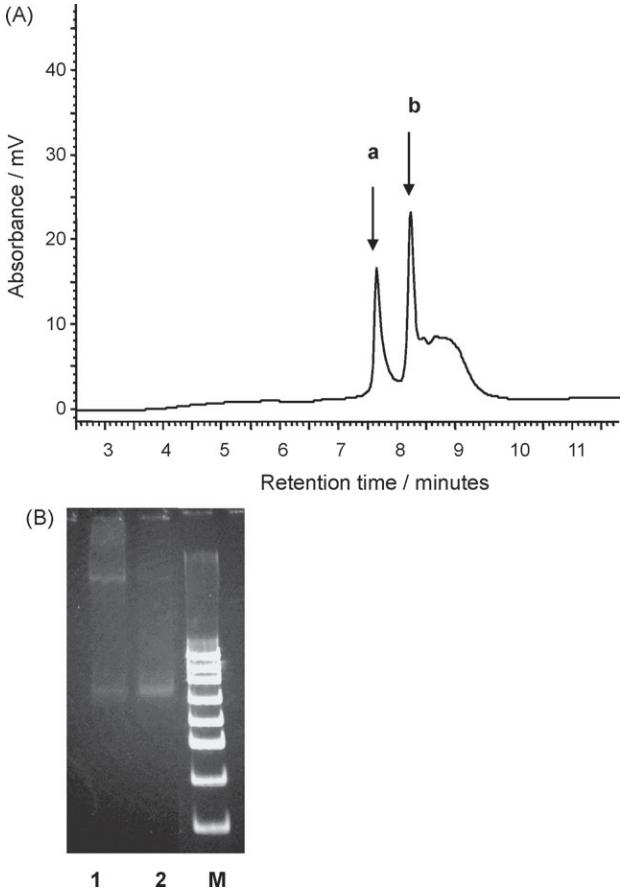

Fig. 6. PAGE analysis of the fractionated hTR species. (A) The hTR species (a) and (b) were fractionated using IP-RP-HPLC in the presence of 1 mM Mg^{2+} at 40 °C. (B) Electrophoretogram showing the analysis of the fractionated hTR samples. Fraction (a) was run in lane 2 and fraction (b) run in lane 1. Lane M contains a 100-bp DNA ladder.

by RNA polymerase II and processed at its 3′-end to produce a transcript of 451 nucleotides (nt) in length [25–27]. The telomerase holoenzyme may act as an independent multimer (a dimer) with at least two active sites [28–30]. It has been shown that human telomerase forms an active complex containing two homotypic hTR molecules per telomerase complex [31]. More recently, it has been demonstrated using native PAGE and agarose gel electrophoresis that hTR can dimerise *in vitro* [32,33]. Ren et al. further demonstrated using single molecule fluorescence coincidence, the multimerisation of hTR in solution and through mutagenesis and oligonucleotide blocking experiments, defined the internal J7b/8a loop as the site of the RNA:RNA interaction [33]. Recent structural studies have provided further insight regarding the structure and function of hTR [34,35].

The analysis of hTR *via* non-denaturing PAGE is shown in Fig. 5A. The electrophoretogram shows the presence of two major RNA species, predicted to be the monomer and dimer of the 451 nt hTR. These results are consistent with previous results using non-denaturing PAGE and agarose gel electrophoresis [32,33]. Analysis of the hTR species using RNA chromatography at temperatures between 30 and 75 °C results in the appearance of a single peak (data not shown). This implies that hTR still runs as a single species (monomer) under non-denaturing conditions. The folding of RNA lent cations [36] under routine chromatographic conditions no metal ions are present in the running buffers (most metal ions are in fact detrimental to the columns used here). Moreover, the ion pair reagent is associated with the negatively charged phosphate backbone of the nucleic acid. To stabilise the dimeric (or multimeric) hTR molecules as observed under non-denaturing PAGE conditions, magnesium ions were included in the chromatography buffers (magnesium has no effect on column stability). The hTR was folded (see Section 2) and subsequently analysed using RNA chromatography in the presence of 1 mM Mg^{2+} over a range of temperatures (40–70 °C), see Fig. 5B. The chromatogram shows that at elevated temperatures (60–70 °C), the hTR fragment runs as a single species (monomer). However, as the temperature is decreased (40–50 °C) two peaks emerge. These results indicate the presence of at least two different RNA species consistent with those observed under non-denaturing PAGE. Further analysis was performed to determine whether the two species separated by chromatography in the presence of magnesium are the multimeric species observed in PAGE. The two hTR species were recovered following chromatography, precipitated and analysed using non-denaturing PAGE. The results are shown in Fig. 6. The early eluting RNA species (a) when analysed using PAGE migrates as the monomer and the later peak (b) migrates as the multimeric species. These results demonstrate that in the presence of Mg^{2+} ions the dimer (or multimeric) hTR RNA species, is stabilised and elutes later than the monomer. Following fractionation, it can also be seen that in the case of the later eluting RNA species (b), when analysed using non-denaturing

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